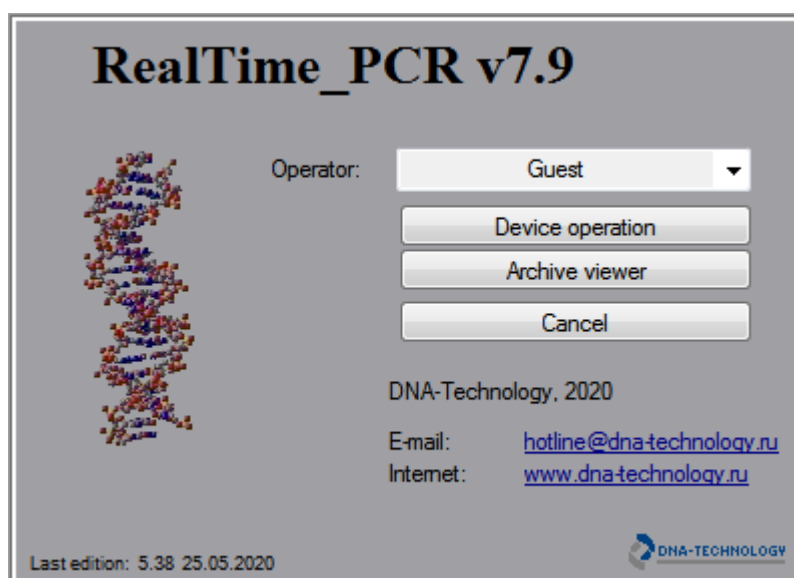# Operation Manual

PART II

RealTime_PCR

Software guidance for

DTprime and DTlite

Real-Time PCR devices

DNA-Technology, Research & Production LLC

Protvino

Contents

1 Introduction

The RealTime_PCR software is designed to control the detecting thermocyclers DTPrime and DTLite.

The Real_Time PCR software allows creating a RUN and to analyze the results of optical measurements.

The RealTime_PCR software is designed for clinical laboratories usage, that is why the instrument management functions and the results analysis settings are simplified as much as possible. At the same time, if desired, the user can change the parameters of the instrument operation and data analysis with the help of additional settings of the RealTime_PCR software.

2 Purpose and conditions of use

2.1 Purpose of the RealTime_PCR software

The-software is designed to control the operation of DTprime and DTLite Real-Time PCR instruments (hereinafter referred to as device).

The following operations are performed using RealTime_PCR software:

1. Plate protocol creation.

2. Test creation and editing.

3. Creation, launching, and monitoring of the amplification program.

4. Analysis of optical measurements data.

5. Data analysis report.

6. Checking geometrical and optical settings of the device.

7. Settings and diagnostics of the device.

2.2 The main features of RealTime_PCR software

1. The software is designed to control the operation of the devices. The software interface automatically adjusts to the type of the selected device.

2. There are two modes of the software implemented in independent functions: **Device operation** and **Archive viewer** allows simultaneously working with devices (create/start/analyze current RUN) and analyze the results of previous RUN).

3. Ability to operate several devices at the same time: RealTime_PCR software is launched in a separate window to control each of the connected devices – "**multi-windowing mode**". This mode allows monitoring simultaneously the operation of several devices.

4. In case of impossibility to start multi-window mode, the option of working in "**single-window mode**" is also possible. This is the mode of the RealTime_PCR software, which allows working with several devices in one window. It might be useful in laboratories with several devices, which can not be connected to the computer simultaneously. In this case, the devices are started via the RealTime_PCR software, and then disconnected from the software and run autonomously.

5. The possibility of introducing a vertical or horizontal temperature gradient across the matrix for device with a solid matrix.

6. The possibility of independently setting temperature for each of six sections for the device with a 6-sectional matrix.

7. The possibility of introducing vertical gradient or independent temperature settings of each of the three sections for the device with a 3-section matrix.

8. Two alternative methods for analyzing the DNA accumulation curve during PCR: **Threshold (Ct)** and **Geometric (Cp)**, which are based on different approaches to determining the indicator cycle of amplification. By default, when analyzing optical measurements, RealTime_PCR software uses the Geometric method (Cp).

2.3 PC requirements

PC requirements are described in section 1.5 of the Part I of operation manual.

3 Preparing for operation

3.1 The RealTime_PCR software installation

The procedure is described in Section 3.3 of the Part I of operation manual.

3.2 Software update

The RealTime_PCR software is constantly updating by DNA-Technology. The latest version of the RealTime_PCR software could be downloaded on the website:

http://www.dna-technology.ru/eng/support/

It is recommended to follow the updates and use the latest version of The RealTime_PCR software.

3.3 Preparing the device for operation

Before the first run of the device, the geometrical settings of the optical unit have to be checked as well as the purity of the wells, the height of the tubes shall be measured, and the exposure of the optical measurements has to be-set.

❖ Optimal exposure values have to be provided by the PCR kits manufacturer. When using the DNA-Technology PCR kits, the factory settings are optimal and preset in the device. When the PCR kits of different manufacturers are used, the exposure values should be checked before each start of the amplification program.

❖ Measurement of the tubes height has to be made in case of using plastic consumables of different producers.

❖ Checking the purity of the thermal block wells has to be carried out periodically to exclude the possibility of obtaining unreliable results as a cause of background fluorescence increase.

The geometric settings of the device's optical unit and exposure should be checked once during the installation procedure. It might be necessary to change these settings when changing a manufacturer of

commercial kits and also in case it is stated in the instructions for kits or in case of any doubt the device settings are correct.

3.4 Description of the RealTime_PCR software menu
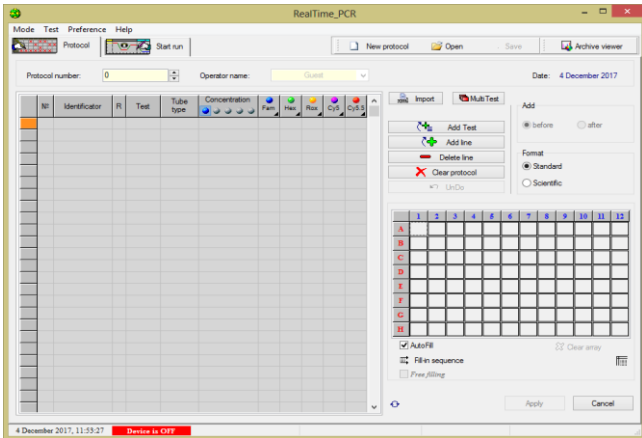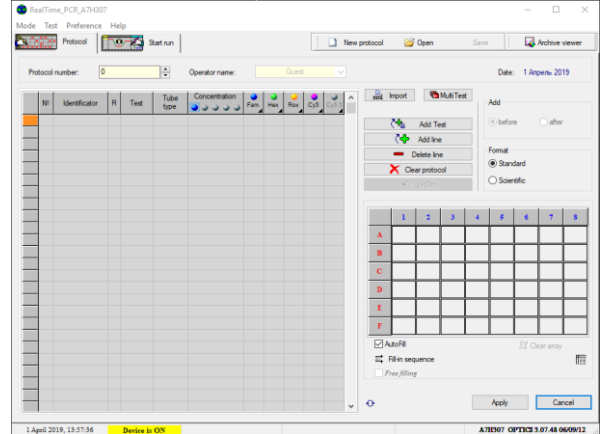
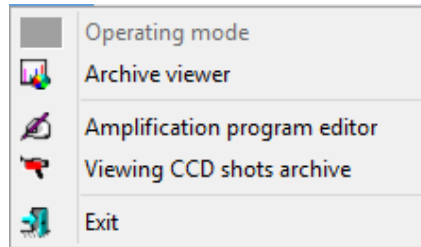(For DTprime)   (For DTlite)

Fig.1 The RealTime_PCR window for the Device operation mode

The main menu of the RealTime_PCR software (Fig.1) consists of four sections:

- **Mode**
- **Test** (available in the "Device operation" mode only)
- **Preference**
- **Help**

1. **Mode** section is designed to control the operation of the software and includes the following commands:

❖ **Operating mode** – the command is active in the *Archive viewer* mode and is intended for switching to the mode *Device operation*.

❖ **Archive viewer** – the command is active in the mode *Device operation* and is intended for switching to the mode *Archive viewer*.

❖ **Amplification program editor** – opens the *Amplification program editor* window.

❖ **Viewing CCD shots archive** – opens the window *Viewing CCD shots archive,* which allows viewing the video of a matrix with tubes for each measurement, provided that after starting the amplification program a field *View the current video image* has been ticked in the *Current image* window.

❖ **Exit** – exit from the software with the closing of the corresponding window.

2. Section **Test** is intended for creation and editing tests and includes the following commands (available in the mode "Device operation" only):
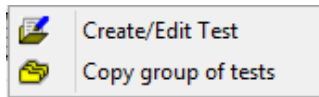
❖ **Create/Edit test** – displays *Create/Edit test* window to create a new test or edit an existing one.

❖ **Copy group of tests** – displays the window to copy the tests of the operator specified in the left part of the window, or from ini files with test parameters for Real_Time PCR software (ini-files, which are provided by the kits manufacturer) to the operator selected in the right part of the window (or to all operators).

3. Section **Preferences** is intended to display and edit the device settings. The "Instrument operation" mode includes the following commands:
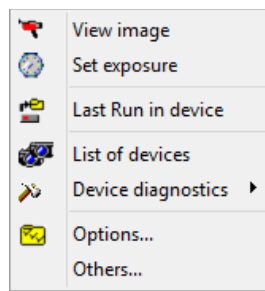
In the "archive view" mode this section looks as follows:
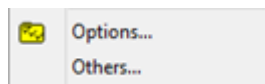
❖ **View image** – displays *View image* window in the process of setting up and diagnosing the device (detailed description, on p.65, section 6 Settings and diagnostics of the device).

❖ **Set exposure** – displays *Set exposure* window in the process of setting up and diagnosing the device (detailed description, on p 65, section 6 Settings and diagnostics of the device).

❖ **Last Run in device** – reading the results of optical measurements from the last run on the device.

❖ **List of devices** – displays *List of devices* window with the function of connecting or disconnecting them.

❖ **Device diagnostics** – sub-menu of the **Preference,** intended for service maintenance and diagnostics of operation of the device's various systems:
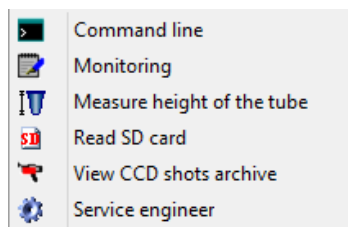
– *Command line* – displays the window where the diagnostics of the optical, temperature, motor and display modules of the device are performed;
– *Monitoring* – opens the window for continuous display of service information necessary for diagnostics of the device operation;

- *Measure height of the tube* − starts the procedure for measuring the height of the tubes while maintaining the obtained value for further work with the device;
- *Read SD card* − displays window for diagnosing the operation of the SD card;
- *View CCD shots archive* − opens the window *Viewing CCD shots archive*, which allows viewing the video of a matrix with tubes for each measurement, provided that after starting the amplification program a field *View image* has been ticked in the *Current image* window;
Service engineer − displays the window for optical calibration of the device (used by service engineer only);

❖ **Options** – displays *Options* window to set user and analysis parameter settings.

❖ **Others** – displays *Others* window to change the parameters of the analysis of optical measurements and adjust advanced settings for the data processing and contain the following options:

Attention! Settings for operating with optical measurement data set by default are optimal for working with commercial kits. These settings are stored in hidden system files and do not change when the RealTime_PCR software is updated.

Type _AnalyseExpo: Selecting the type of "stitching" of optical data when the saturation level is reached: linking the two exposures or selecting one of the two (Fig.2).
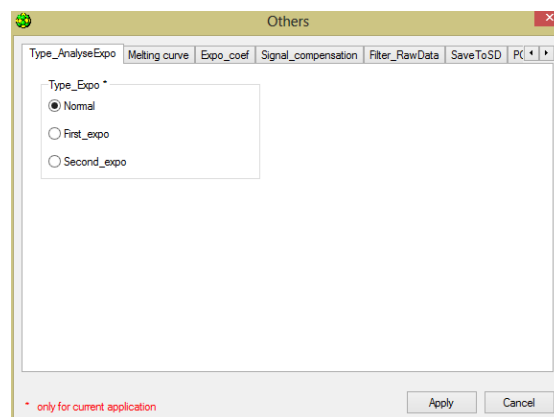

Fig.2 Tab «**Type _AnalyseExpo**»

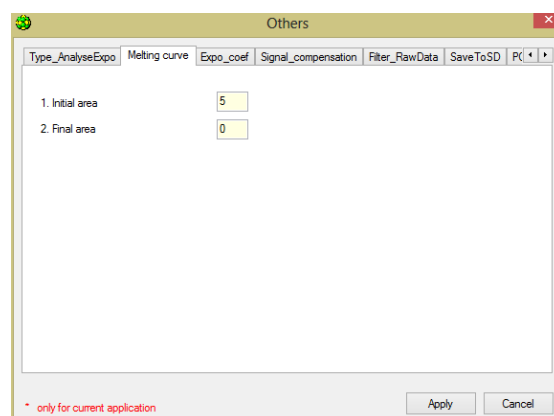**Melting curve**: Determine areas of "smoothing" of the melting profile (Fig.3).

Fig.3 Tab «**Melting curve**»

Expo_coef: a factor that determines how many times the second exposure is less than the first (Fig.4).
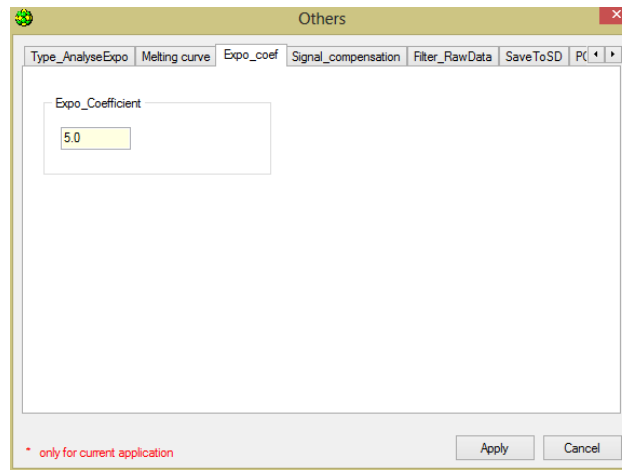
Fig.4 Tab «**Expo_coef**»

Signal_compenstaion: determine the existence of algorithms for "color" compensation between optical channels, correction of the optical signal along the thermal block and between channels (Fig.5).
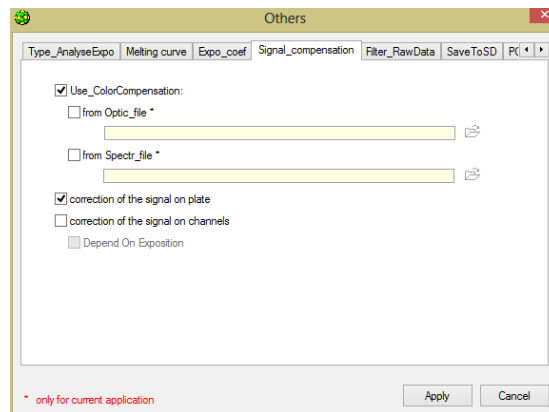

Fig.5 Tab «**Signal_compenstaion**»

Filter_RawData: the presence of a digital filter in the analysis of the original data and the use of additional analysis on the final "buildup". The borders of validity in describing the PCR curve with the sigmoid (Fig.6).
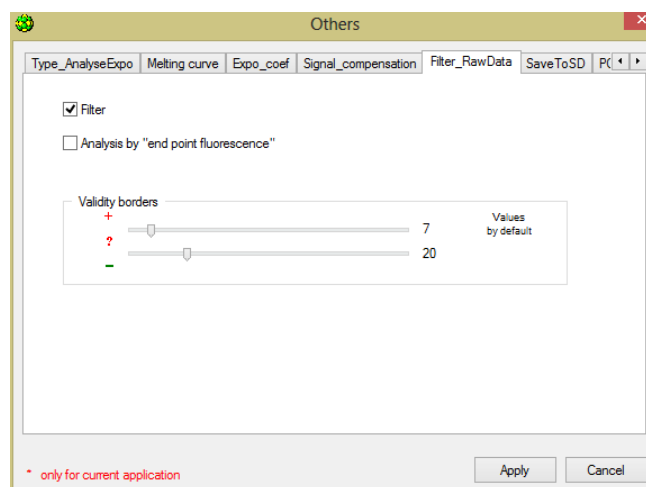

Fig.6 Tab «**Filter_RawData**»

Save to SD: saving optical measurement data on an SD card (not recommended) (Fig.7).
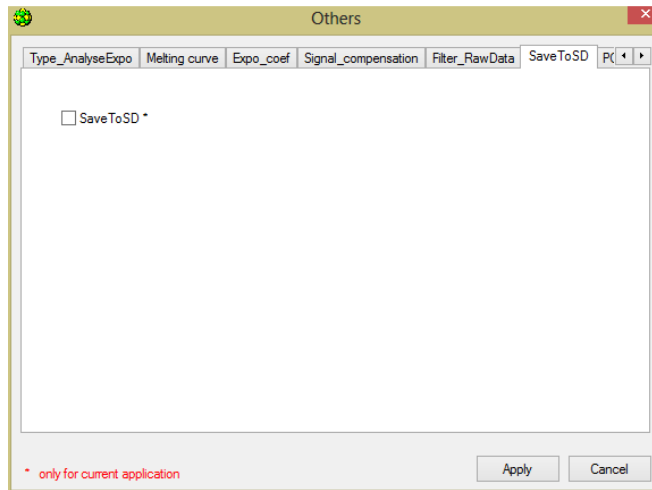
Fig.7 Tab «**Save to SD**»

PCR_settings: define the left boundary of the search for the "Take_of point" in the case of PCR analysis. Coefficient of reduce Cp relative to the location of the maximum of the second derivative in PCR sigmoid (Fig.8).
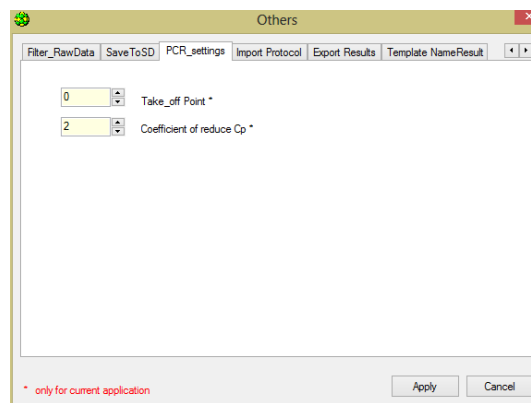

Fig.8 Tab «**PCR_settings**»

Import protocol (RUN) from LIS (Laboratory Information System) (Fig.9).
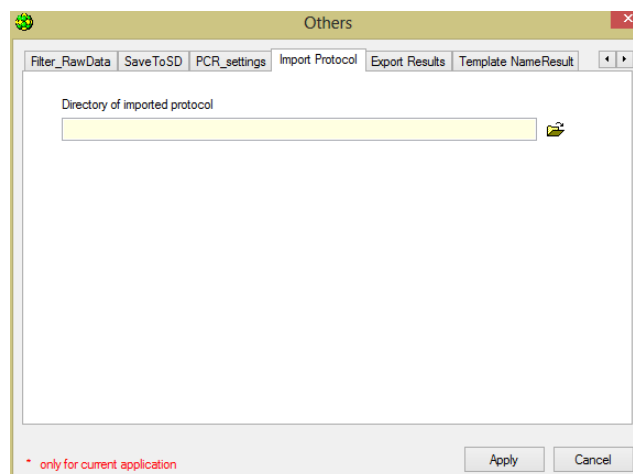

Fig.9 Tab «**Import protocol (RUN)**»

Export results: the general settings for the export of results are defined, as well as the features of the report output in the LIS (Fig.10).
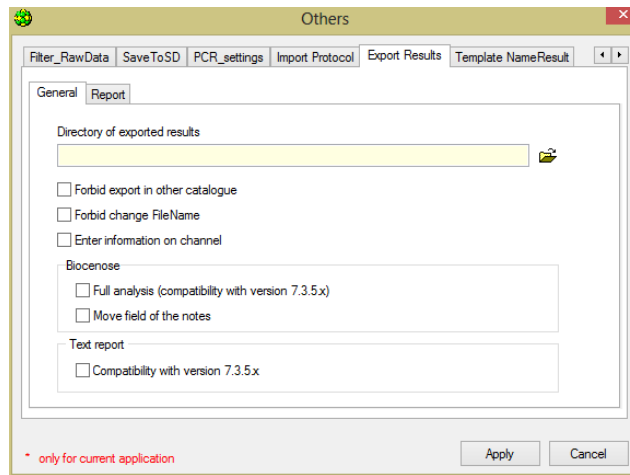
Fig.10 Tab «**Export results**»

Template NameResult: setting the name of the protocol (RUN) to be saved according to a predefined pattern (Fig.11).
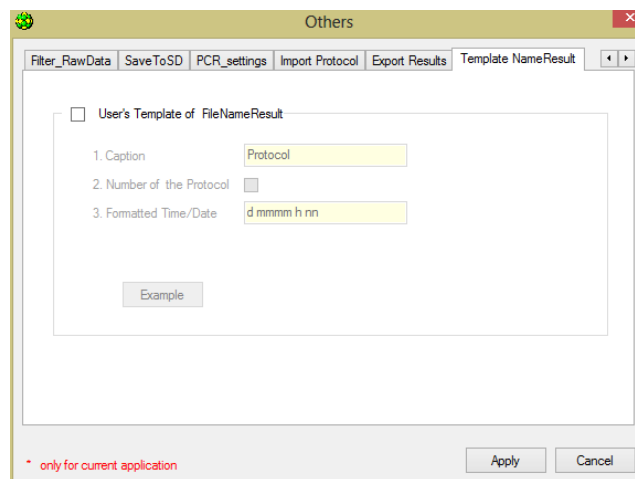
Fig.11 Tab «**Template NameResult**»

4. The **Help** section displays information about the RealTime_PCR software and describing its current version:
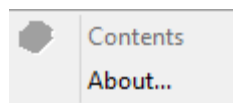
3.5 Running the RealTime_PCR software

The RealTime_PCR software can be launched in two ways:

1. Use the **Start** menu of the Windows operating system:

Start → **All** Programs →**RealTime_PCR** → **RealTime_PCR**

2. Use the icon on the desktop (when the software is installed, this icon is placed on the Desktop automatically).

After the software is launched, a window will appear on the screen for selecting the operating mode of the software (Fig. 12).

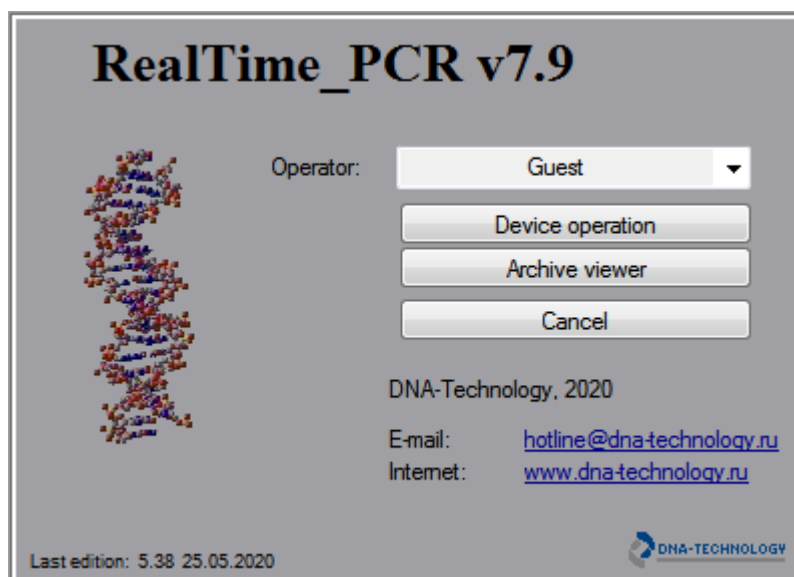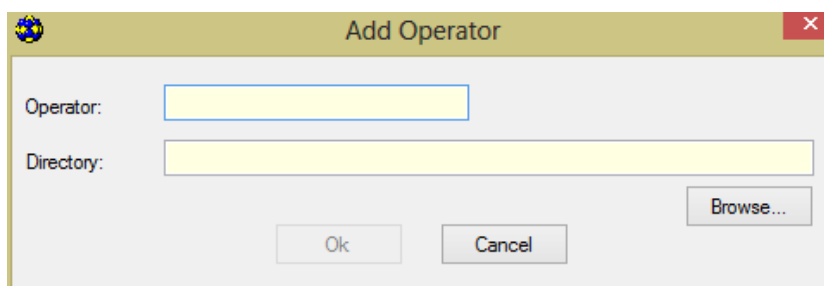Fig.12 The window for selecting the operating mode of the software

The following actions could be performed in this window:

  1. Select an existing operator or add a new operator.

Select an existing operator from the **Operator list**. To add a new operator select **Add operator** item from the **Operator list**. When selecting **Add operator** item, the **Add operator** window appears on the screen (Fig. 13).

Fig.13 Add Operator window

In this window, the fields **Operator** and **Directory** shall be filled in. Enter the operator name in the **Operator** field.

Path to the operator's working directory on the hard disk of the computer in the **Directory** field shall be specified. To select a folder use the **Browse** button. While working under this operator, all files (plate protocols, amplification programs, RUNs) will be stored in the specified folder, unless otherwise indicated.

Press **OK** button to save the specified data and return to the window for selecting the operating mode of the software.

Note: It is not recommended to create a working folder of the operator on the system hard disk, instead of it use any other hard disks of a PC for files' storage. While working with the device under the **Guest** operator, the RUNs will be stored by default in the RealTime_PCR software folder. It is not recommended to operate under Guest operator option.

2. Choose one of two possible modes for operation with the software:

Mode **Device operation,** allowing:

- create and edit tests;
- create and edit amplification programs;
- create and edit plate protocols for upcoming RUN;
- monitor the progress of the amplification program;
- carry out diagnostics and settings of the instrument.

Mode **Archive viewer**, allowing:

- to analyze the optical measurements data from previous RUNs;
- form reports on the results of measurements.

Note: It is possible to work simultaneously in two modes (for example, analyzing the data obtained earlier during the execution of amplification programs by devices, or preliminary analysis of data obtained during the current run).

4 Device operation

4.1   Device operation mode

4.1.1 Mode assignment

Device operation mode of the RealTime_PCR software is intended to create the plate protocols, create/copy tests, create amplification programs, conduct the amplification using the devices and configure device technical parameters. (Fig.14).
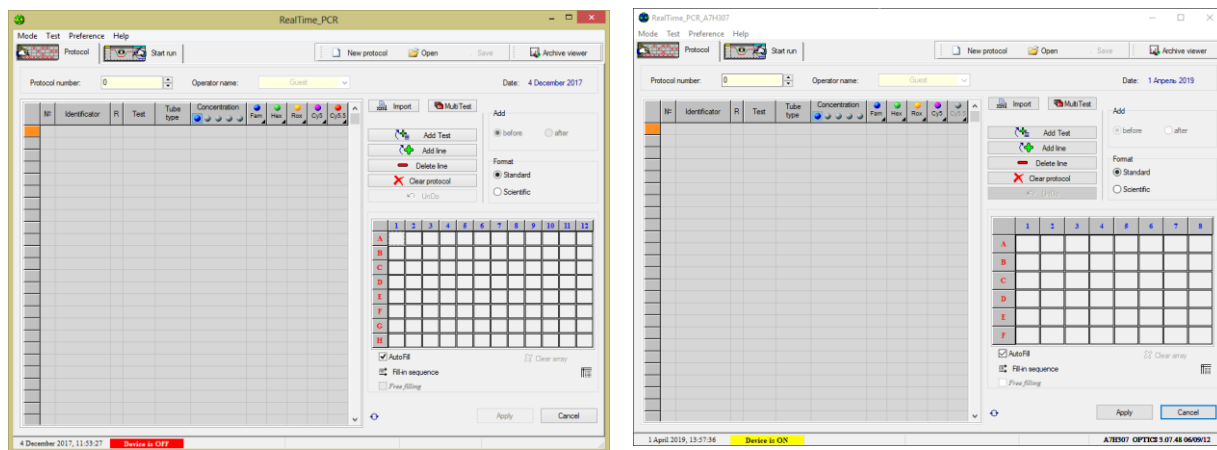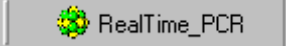

(For DTprime)   (For DTlite)

Fig.14 RealTime_PCR in the Device operation mode

To switch to this mode, use one of the following methods:

Select **the Device operation** mode when you launch RealTime_PCR.

Select the **Operating mode** from **Mode** menu of the **Archive viewer** window.

1. Click on the button [RealTime_PCR] of the **Archive viewer** window.

A dialog window **List of devices** appears on the screen with a list of devices connected to the computer (Fig.15).
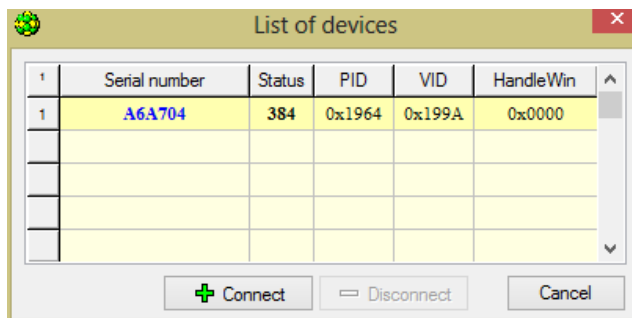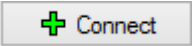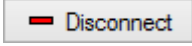

1	Serial number	Status	PID	VID	HandleWin	^
1	A6A704	384	0x1964	0x199A	0x0000	

[+ Connect] [– Disconnect] [Cancel]

Fig.15 List of devices window

To work with a specific device, select it in the list and then press the button [+ Connect]. The RealTime_PCR software is ready to work with the selected device.

Multi-windowing mode allows working with each device, provided that the RealTime_PCR software is launched in a separate window by one of the methods described in section 2.2 item 3. This mode allows simultaneously monitoring the operation of several devices.

Note: In case several devices cannot be connected to the computer at the same time, it is possible to work with these devices through a single-window mode. In single-window mode, after starting the amplification program in the device, disconnect one using the button [– Disconnect] and connect another device.

4.1.2 Operating in the Device operation mode

Consists of the following steps:

1. Creating **a plate protocol** and setting the amplification parameters.
2. Start the amplification program.

4.1.3 Plate protocol

When working with devices frequently, it is necessary to create plate protocols. It shall include the following information:

1. Targets to be determined (one or more, depending on the purpose of the kit).
2. Name (identificator) of the samples.
3. Temperature regime of amplification (amplification program).
4. Type of analysis of the results.
5. Types of fluorophores (fluorescent dyes) used for certain targets (specific target, internal control, etc.).
6. The volume of the reaction mixture.
7. Types of samples used: unknown samples, controls, calibration samples ("standards"), their quantity and concentration (for "standards").
8. Location of tubes in the thermal block matrix.

9. If necessary, additional settings.

After completion of the amplification program, the data analysis contains optical measurements information in addition to the above mentioned settings and stored as a file located in the folder of the operator, unless otherwise indicated.

To view the data analysis after the amplification is completed, open the saved RUN file.

When commercial kits are used and similar studies are conducted, the parameters of the plate protocols are standard and differ from each other, usually in the number of samples and their placement in the thermal block of the device.

To simplify the plate protocol creation procedure, it is recommended to save the standard plate protocol parameters in the form of a **Test**.

4.1.4 Test

The set of all RUN parameters, that is necessary for conducting PCR and subsequent analysis of the results is described as a Test.

Test includes the following RUN parameters:

1. Targets to be determined (one or more, depending on the purpose of the kit).
2. Temperature regime of amplification (amplification program).
3. Type of analysis of the results.
4. Types of fluorophores (fluorescent dyes) used for certain targets (specific target, internal control, etc.).
5. The volume of the reaction mixture.
6. Data on the number of control samples, standards, and their concentrations.

The RUN can consist of one or more Tests having one amplification program and one type of analysis.

The Test can be created once and remain in the list of available Tests for a certain Operator.

To add a Test to the list of operator Tests, the procedure of creating Test using the option **Create**/**Edit test** has to be performed, or by using the procedure **Copy group of tests**.

Copying a group of Tests involves the presence of ini files with all parameters for the RUN (ini-files, provided by the DNA-Technology company). When creating a Test using an ini-file, the final stage of process after the completed RUN is the formation of a result form using the Form of the answer .

When preparing to a RUN, it is sufficient to indicate **Test** in the **Add test** window (all the necessary parameters will be loaded automatically), the number of samples to be examined and their placement in the thermal block of the device should be indicate by Operator.

The RUN can be created without using the option **Test**, in this case, all necessary information shall be entered in the plate protocol fields manually. When operating in this mode, Operator cannot select the type of analysis. *Note:* The default type of analysis for such RUN creation is **Qualitative**.

Attention! It is not recommended to create a protocol without using the **Test** procedure since the **Analysis parameters** window will not be active, and the optical calibration coefficients of the device will not be used (see Section 6.4).

4.1.5 Creating/editing tests

4.1.5.1 Creating a new test with the option Create/Edit test

1. To create a new test, click the menu **Test** of the RealTime_PCR window and choose the command **Create/edit test**. In the appeared window (Fig.16) click **Create a new test DNA-Technology, Research & Production LLC**
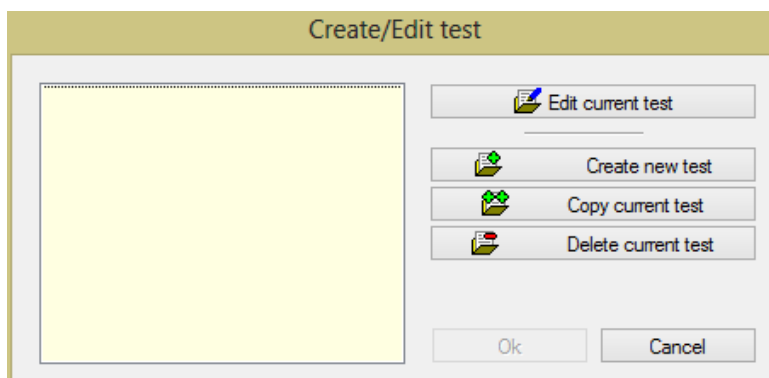
Fig.16 Window «**Create a new test**»

2. Then enter the name of the new Test in the next window (Fig.17), if necessary activate the **Multi-test mode** option (see section **Multi-test mode**) and click on **OK** button.
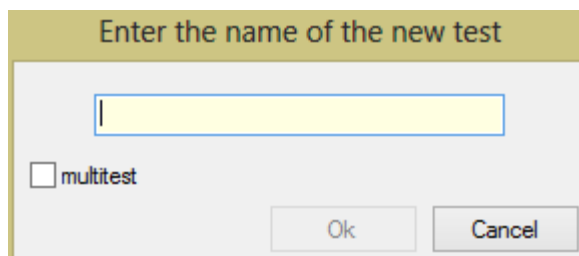
Fig.17 Window «**Enter the name of the new test**»

3. The Test window appears. Enter Test parameters in the appropriate fields in the Test window (see **Test Parameters** section).

4. Press OK button to save the specified parameters and exit the Test window.

4.1.5.2 Editing a Test

1. To edit the Test in the window **Create/Edit test** select the Test and click **Edit current test**.

   The Test window appears (Fig.18).

2. Change the required fields in the Test window (see section **Test Parameters**).

3. Press OK button to save the specified parameters and exit the Test window.

4.1.5.3 Test Parameters

The description and Test parameters are indicated in the **Test** window (Fig.18).

Drop-down list of **Test** is intended for selecting a Test. When selecting an item from the list in the **Test** window the parameters of the selected test are displayed.

In the **Description** field, it is recommended to specify the purpose and features of the test.
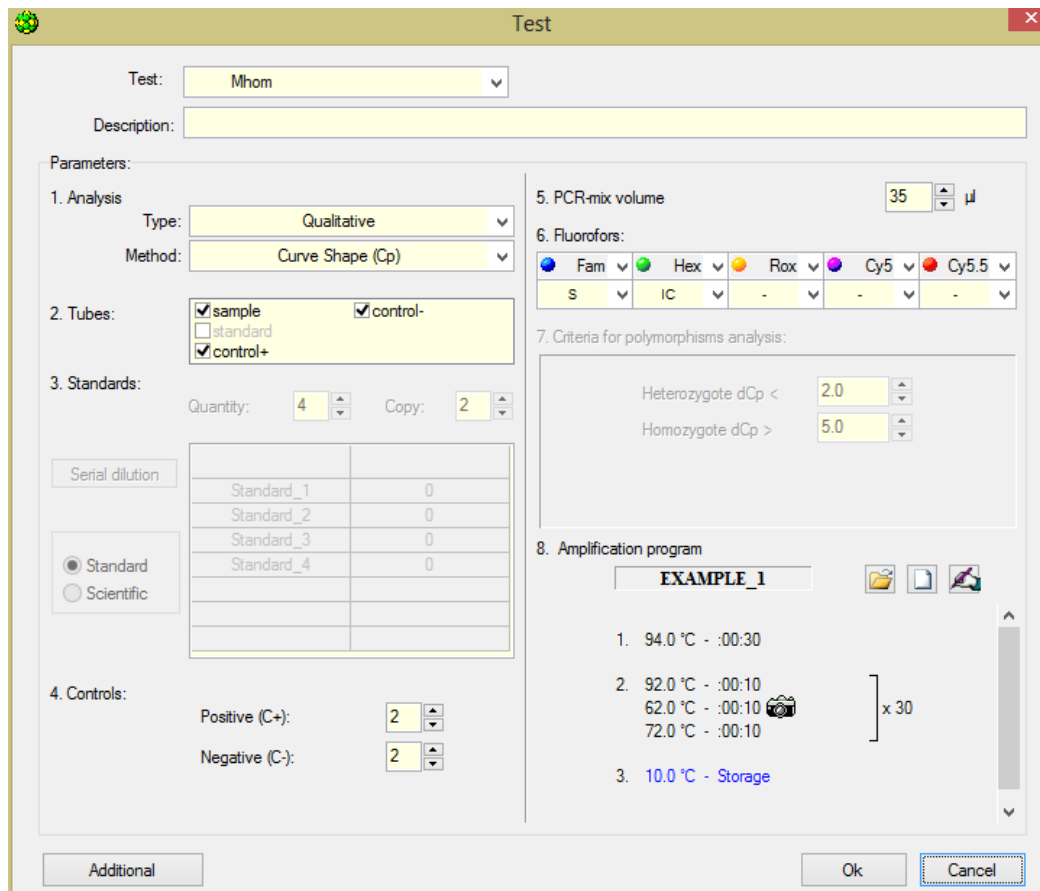

Fig.18 The Test window

Then specify Test **parameters** necessary for conducting PCR and analyzing the results.

Note: If commercial kits are used, the required parameters are specified in the kits' instruction for use.

Parameters description:

❖ **Analysis**

In **Analysis** section, select the Analysis **type** and Analysis **method** options.

▪ *Type*

For more information on the types of analysis see Appendix 2.

▪ *Method*

The following methods are implemented in the software:

− *Geometric (Cp)* − determination of the indicator cycle on the basis of mathematical analysis of the shape of the amplification curve;

– ***Threshold (Ct)*** – determination of the Ct (*threshold cycle*). This method is based on conducting a threshold line and determining the cycle number for each sample, at which the amplification curve crosses the threshold line.

*Note***:** To change the values of the threshold cycle, press the button **Additional** and change it in the opened window **Parameters of the analysis** "point 2.Threshold value". The value of the threshold line can also be changed using the mouse in the graphical representation window.

For more information about advanced options, see «**Parameters of the analysis**».

❖   **Tubes**

The types of samples that are used in the analysis should be marked.

– ***Sample*** – the test sample;

– ***Standard*** – calibration sample required for quantitative analysis;

– ***Control +*** – positive control sample;

– ***Control -*** – negative control sample.

❖   **Standards**

When choosing the **Quantitative with standards** type of analysis it is necessary to describe the used calibration samples ("standards").

To do this, the following information has to be provided:

– fill in the ***Quantity*** field – indicate the number of different concentrations of calibration samples ("standards");

– fill in the ***Copy*** field – indicate the number of repetitions of each concentration of calibration samples ("standards");

– fill in the **table of "standards" values** (specify the name and concentration of each "standard").

The number of rows in the table corresponds to the number of variants of "standards". For each "standard" you need to specify a name (by default this is "Standard_1", "Standard_2", etc.) and specify the value (concentration of the "standard"). Units (copies, picograms, IU) can be selected from the drop-down list in the title bar of the right column of the table.

The table of "standards" values can be filled manually or automatically. For automatic filling, press the button **Serial dilution**, in the appeared window fill in the fields **Initial value** and **Dilution step** and click the button **Apply**.

Data in the table of "standards" values can be presented in two formats: standard and scientific. Select the presentation format using the switch **Format**.

Example. Indication of values in the standard and scientific format. *Note:* the degree of 10 (order) of the value is indicated after the letter "E" in scientific format).

Standard	Scientific
5	5,00E+00
15	1.50E+01
250	2.50E+02
9,500	9.50E+03
18,500,000	1.85E+07

❖ **Controls**

In **Controls** section, it is necessary to indicate the number of tubes used in the Test with control samples.

– *Positive (C+)* – the number of tubes with positive control samples.

– *Negative (C-)* – the number of test tubes with negative control samples.

Note: The number of tubes with calibration and control samples, if necessary, can be changed during the compilation of the plate protocol (see Section 4.2 Creating a plate protocol).

❖ **PCR- mix volume**

In **PCR-mix volume** field, it is necessary to indicate the volume of the reaction mixture without taking into account the volume of oil or paraffin (if used).

For the most effective and accurate performance of temperature regimes of amplification, an "active" method for controlling the temperature of the reaction mixture in a tube is used in the device. The method is based on the application of a mathematical model of the reaction tube and requires an accurate indication of the volume of the reaction mixture.

❖ **Fluorophores**

In the **Fluorofors** table the fluorophore and its purpose has to be selected and indicated from the drop-down lists for each channel when analyzing the results:

Specific target – the main, specific signal.

IC – signal from internal control sample.

If some channel is not used, select in the **Fluorofors** table the item **Not detected**.

Note: If R6G dye is used in PCR kit, then while creating (editing) a Test in the HEX channel choose from the drop-down list .
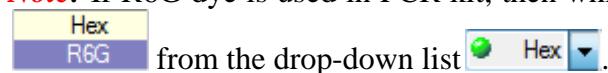
When choosing the **Analysis of polymorphisms (2 probes)** or **Analysis of polymorphisms (MC) auto** or **Analysis of polymorphisms (MC)** type of analysis, it is necessary to specify the allele for fluorophores signal (by default this is "a" and "b").

❖ **Criteria for polymorphisms analysis:**

The section is active when **Analysis of polymorphisms (2 probes)** or **Analysis of polymorphisms (MC) auto** or **Analysis of polymorphisms (MC)** type **of analysis** is selected.

When analyzing **Analysis of polymorphisms (2 probes)**, the results are evaluated by the presence and magnitude of relative displacement amplification curves recorded in one tube for two fluorophores (dCp or dCt).

In the **Analysis of polymorphisms (MC)**, melting type, the interpretation of the results is based on the correspondence of the melting temperatures of the signal probes to the temperatures indicated in the **Criteria for polymorphisms analysis** field.

In the **Analysis of polymorphisms (MC) auto** type, the interpretation of the results is based on comparing the characteristics of reference melting curves contained in the library file, and melting curves obtained in the present experiment.

❖   **Amplification program**

In section **Amplification program** an amplification program has to be specified in one of the following ways:

− select the previously saved amplification program using the button ⬛;
− create a new amplification program using the button ⬛;
− edit the current amplification program using the button ⬛.

The selected amplification program will be displayed in this section. For more information about creating an amplification program, see section 4.1.6 "Creating and editing amplification programs".

❖   **Additional**

When you press the **Additional** button the **Parameters of the analysis** window opens.

See the description of additional analysis parameters in section 8 **«Parameters of the analysis»**.

Attention! This section is intended for advanced users only. In most cases, there is no need to change the default values of the analysis parameters. Changing the analysis parameters stored in the software, may lead to incorrect interpretation of the obtained data analysis.

4.1.5.4 Create a new Test using the Copy group of tests option

Creating a Test is also possible using ini files with test parameters for the Real_Time PCR software, if they are provided by a kits manufacturer (ini-files are provided by DNA technology). Files with test parameters already contain all necessary information that is entered in the **Test** window. When copying tests, the information is saved in the list of operator tests.

In the main menu of the **RealTime_PCR** software select the **Test**, See (Fig.19):
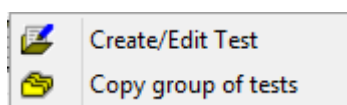
Fig.19 Step to creating a test

Click **Copy group of tests** in the drop-down list. The **Copy group of tests** window opens (Fig.20):
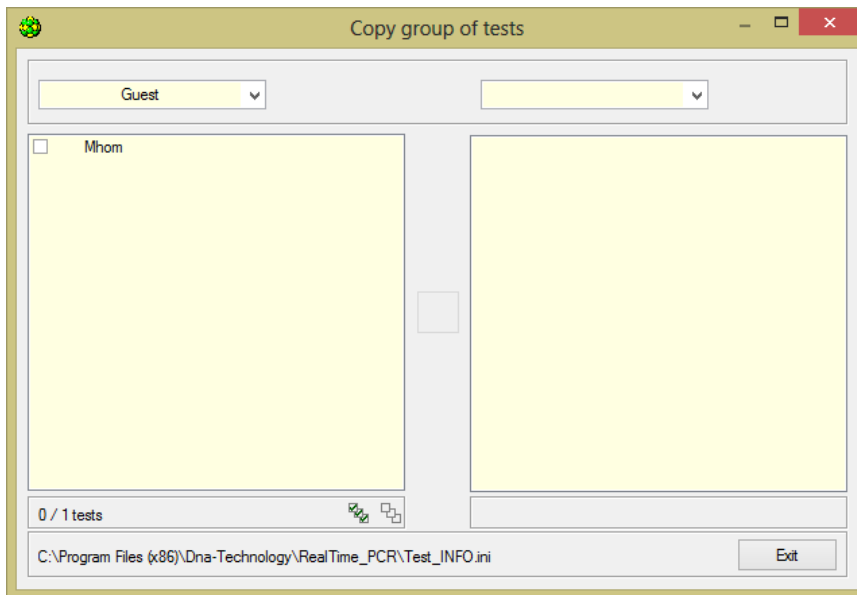
Fig.20 Window « **Copy group of tests** »

In the left part of the opened window, select the item "from ***.ini file**", see (Fig.21):
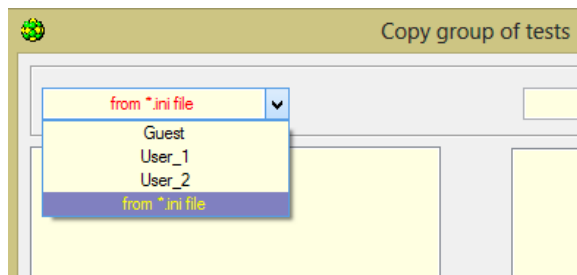
Fig.21 Select the item

1. In the opened window select the necessary ini-file, click "Open".
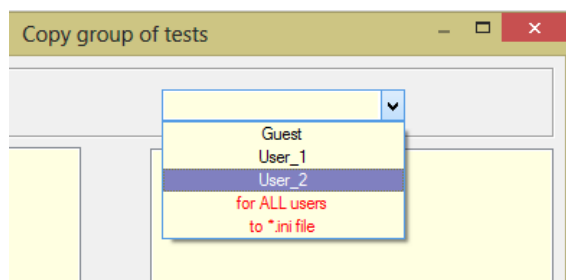2. In the right part of the «Copy group of tests» window, select the operator who will use this test,see (Fig.22):

Fig.22 «Select Operator»

Click ☑ to choose the necessary test from the list on the left or select all tests using the button ▨

and click ➡. After that, the test should move to the right list and the following message will be displayed, see Fig.23.
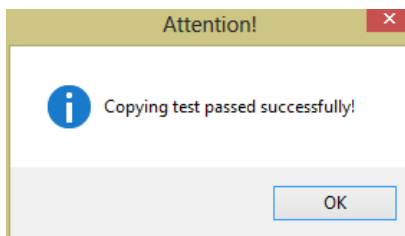
Fig.23 The message about the successful completion of the test copy

4.1.5.5 Multi-test mode

Multi-test mode is the option that allows combining several tests in one RUN with the same amplification program and analysis type, creating **batch studies**.

Multi-test mode can be used for the following **analysis types: Analysis of polymorphisms (MC), Relative** and **Qualitative**.

While creating **batch analysis** (see section 4.1.5 Creating a new test using the Create/Edit test option), the option **multi-test mode** has to be activated, See (Fig 24).
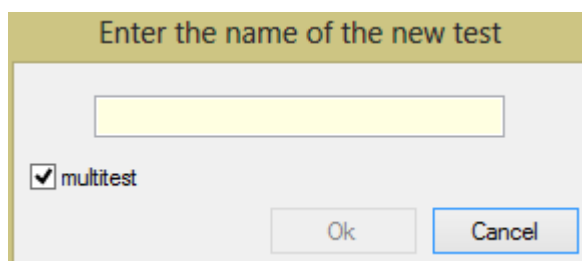
Fig.24 Multi-test mode option selection

In the opened window **Test** (Fig.25) the operator can select all previously created tests, selecting from which the operator forms a list of new **batch analysis**. Test list of this **batch analysis** in the required order is displayed in the **Location tests** window, see (Fig.26).
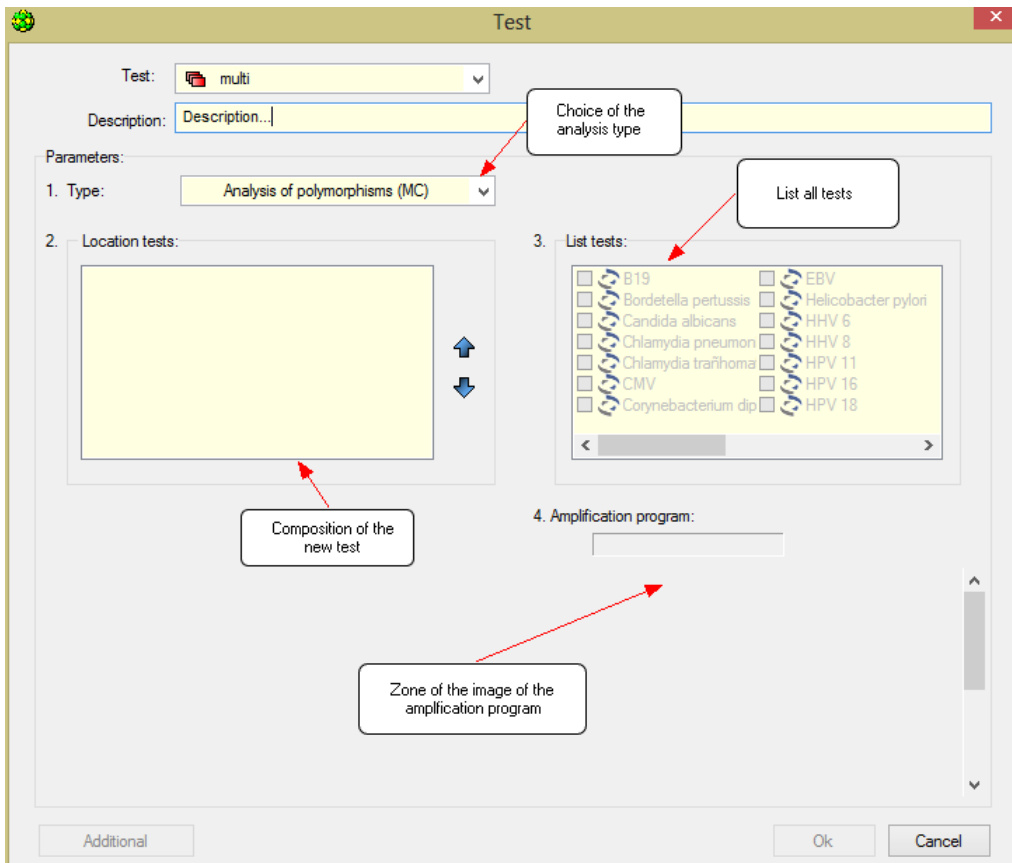
Fig.25 Multi-test test window
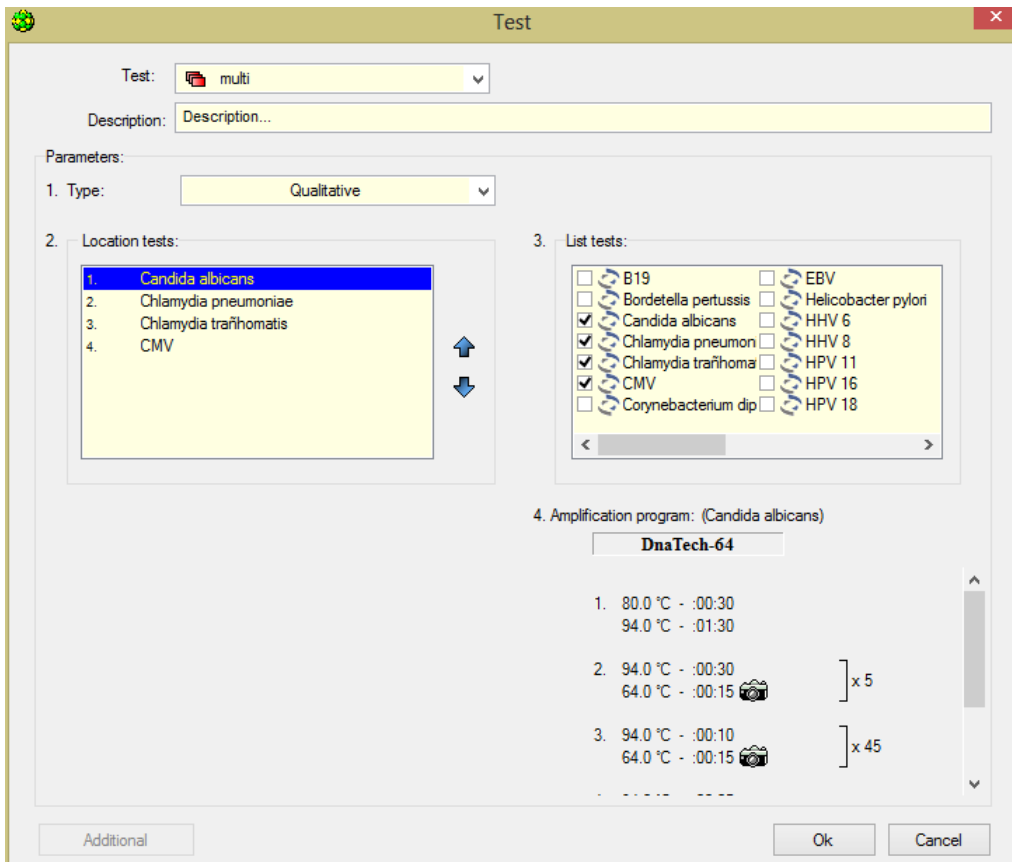
Fig.26 Multi-test mode test window. Creating analysis

The selected tests should have the same amplification program. If a test with another amplification program is added, a warning message will appear, see (Fig.27).
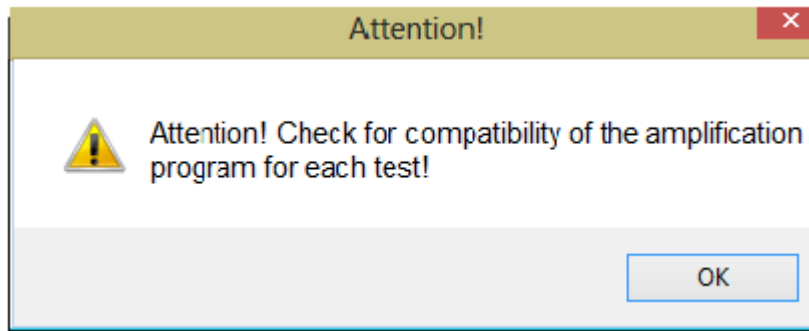
Fig.27 Message about the presence of tests with different amplification programs

After creating a new batch analysis list, click OK. The created Test will be saved in the list of the operator tests and will be marked with an icon ⬛.

When preparing to RUN for batch studies, Operator marks the number of samples in a window **Add test**, there is an indication of the number of tubes for each sample in brackets (Fig.28).
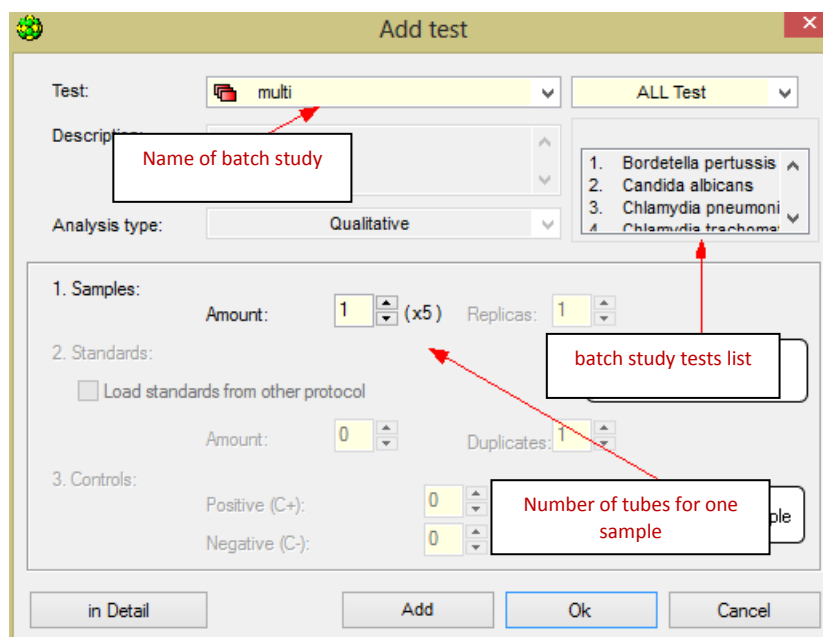
Fig.28 Window **Add test** when working with batch analysis

4.1.6 Amplification program

4.1.6.1 Creating and editing of amplification program

When using the option **Copy group of tests**, the amplification program is loaded into the software automatically.

When creating a test with the option **Create/Edit test**, the amplification program is entered by the operator.
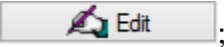
When preparing to RUN without the help of an **Add test** option, the amplification program is created by Operator or added from the list of previously created programs just before each start of the RUN.

4.1.6.2 Creating a new amplification program

There is an option to create a new amplification program at various stages of the **RealTime_PCR** software:

1. When creating (editing) a test with the option **Create/edit test:**
   – open the window **Test** (see the section 4.1.5.1 Creating a test);
   – press the button ◻**Create new program** in point "**8. Amplification program**";

2. When creating an amplification program without using the Add test option:
   – select a tab **Start run** in the **RealTime_PCR** window;
   – press the button ◻**Create new**;

3. When working in **Amplification program editor**:
   The created amplification program will be available for selection before starting the RUN
   – select a tab **Start run** in the **RealTime_PCR** window;
   – press the button ◻ **Edit** ;

   – then select the Menu bar item ◻ Create a new program in the **Amplification programs editor** window.
   – When creating a new amplification program by any of these methods, an **Amplification program template** window appears on the screen (Fig.29). Using the template, specify the structure of the amplification program.

A new amplification program can be created with or without the Amplification program template.

❖   **Create a new amplification program using the Amplification program template**

To create a new amplification program using a template, the following steps shall be proceeded:
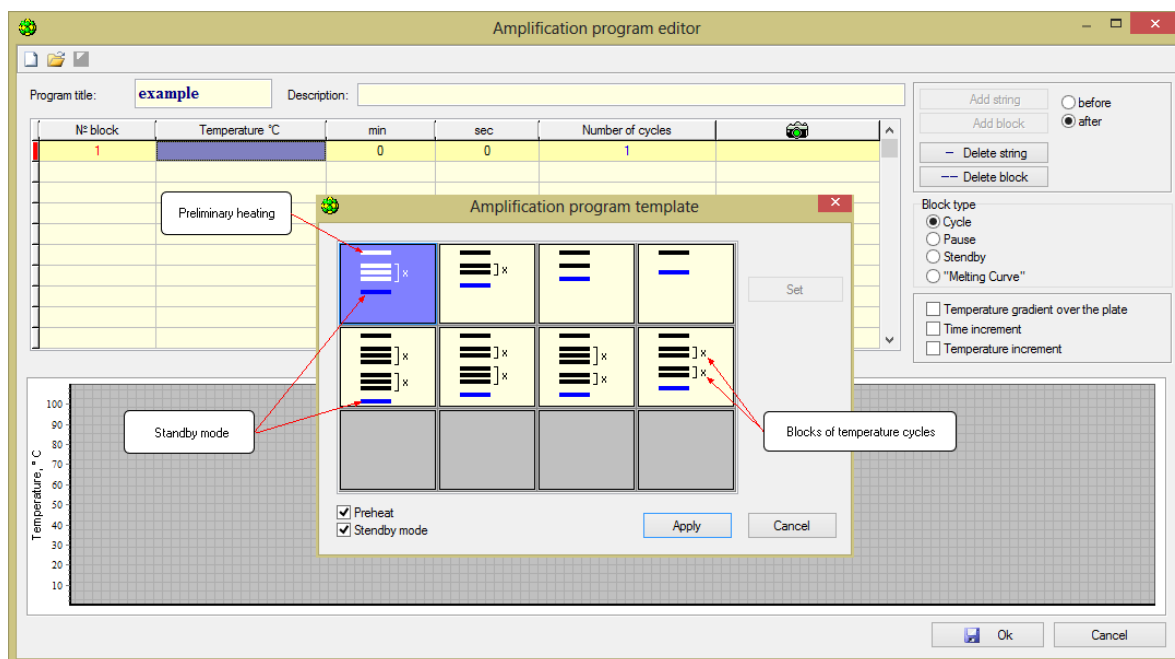
Fig.29 Amplification program template window

1. Choose a **Create new program** command. After that, an **Amplification program editor** window and an **Amplification program** template window will be displayed. The **Amplification**

program template window (Fig.29) schematically shows the parts of the amplification program: two- and three-stage blocks of temperature cycles, preheating and storage modes.

2. Select the template that best matches the structure.

3. If necessary, add **Preheating and Standby modes** (enable the corresponding options).

4. After selecting the most suitable template, press the button **Apply**.

   The information specified by the template will be displayed in the **Amplification program editor** window (Fig. 30).

5. Edit the amplification program:

− enter the name of the program in the **Program title** field (*Note***!** strictly Latin letters and numbers without spaces and symbols);

− fill in the **Description** field if necessary;

− change the required data in the amplification program table.
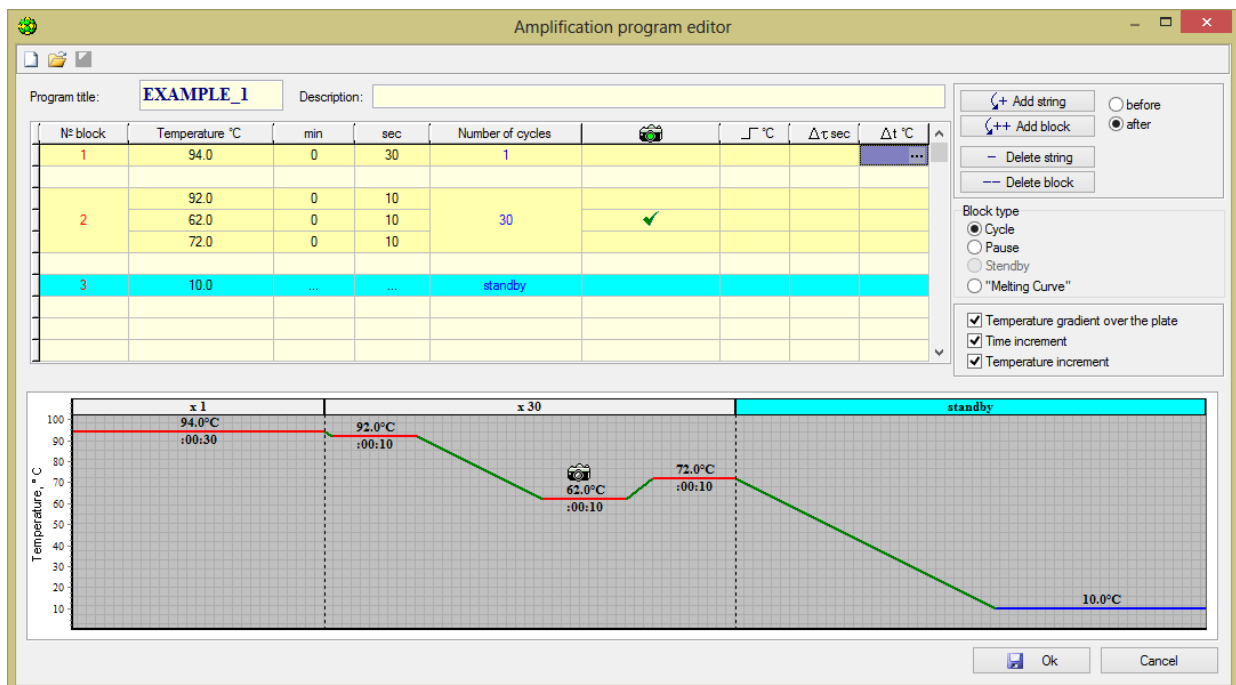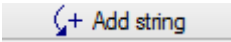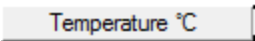
Fig.30 Amplification program editor window

Changing the data in the amplification program table

Each row in the table corresponds to one temperature range of the amplification program. Temperature shelves can be combined into blocks. The number of rows in the block determines the number of temperature shelves at this stage of the program. In the table, the blocks are separated from each other by empty lines.

Adding   〔+ Add string 〕   the temperature shelf is active after completing the editing of other lines only.

In each line for each temperature shelf, the following information shall be edited:

• shelf temperature − 〔 Temperature °C 〕;

- shelf length – [min] and [sec];
- the presence of optical measurements – [📷].

Attention! Only one optical measurement is allowed within one block. Empty fields in the program are not allowed when entering temperature and time values, except for blocks **Pause** and **Standby**.

Adding and deleting blocks are performed with buttons [++ Add block] and [-- Delete block].
The block type has to be specified for each block.

1. **Cycle** (specified by default when creating a block) – sets the mode for repeating the program at this stage until a specified number of cycles is reached.

2. **Pause** – pauses in program execution mode.

3. **Standby** – sets storage temperature after program execution.

   Attention! The «Standby» mode can be set at the end of the program only.

4. **Melting curve** – allows obtaining melting profiles for estimating the lengths and composition of the amplified fragments.

   Selecting the **Melting curve** allows creating the default temperature block of one temperature shelf with an initial temperature of 90 °C, the number of cycles equal to 100 and incremented on each temperature cycle of -0.5 °C. The initial settings of the "Melting curve" block can be edited using the Temperature increment.

The number of cycles has to be specified for each block (Fig.32).

№ block	Temperature °C	min	sec	Number of cycles	📷
1	94.0	0	30	1	
	92.0	0	10	30	
2	62.0	0	10		✔
	72.0	0	10		
3	10.0	...	...	standby	

Fig.32 Example of entering the number of cycles

If necessary, the addition parameters can be specified for each temperature shelf:

- temperature gradient over the plate (temperature drop across the matrix), displayed in the amplification table in the column indicated by [⌐ °C];

- time increment, displayed in the amplification table in the column indicated by [Δτ sec];

- temperature increment, displayed in the amplification table in the column indicated by [Δt °C].

To display the specified parameters in the table, enable the corresponding options (Fig.33).
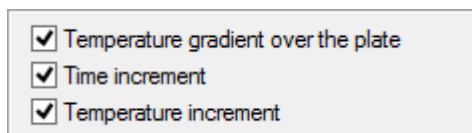
Fig.33 Advanced setting

Editing of these parameters is described in the relevant sections of this manual.

During creating/editing of amplification program its **graphical representation** is displayed at the bottom of the **Amplification program editor** window (Fig.34). With any change in the temperature value shelf, its duration, the number of temperature blocks, the number of cycles, the change in the block type, etc., are displayed in the graphic representation of the amplification program.
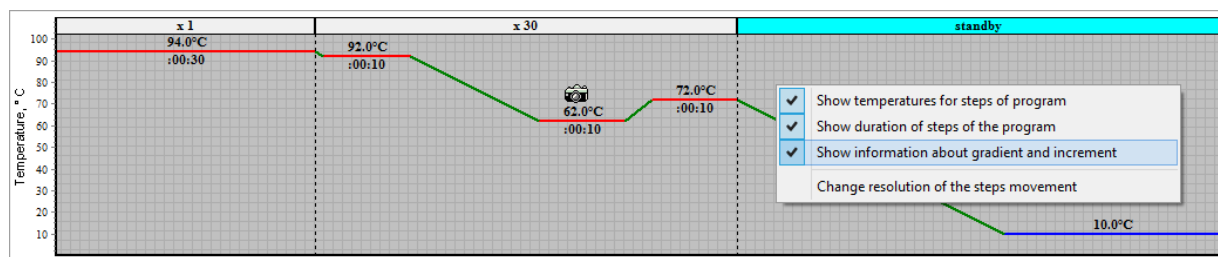

Fig.34 Graphical representation of the amplification program

The temperature shelf can be changed by editing the graphic image of the amplification program. To do this, bring the mouse cursor to the temperature shelf (the mouse cursor becomes) and, while holding down the left mouse button, move (in the vertical direction) the temperature shelf to the necessary temperature.

The graphic representation of the amplification program can be changed using the context menu, which can be opened by the right mouse button, and contains the following items:

- show temperature for steps of program;
- show duration of steps of the program;
- show information about gradient and increment (temperature drop across the matrix, increment by time and temperature);
- change resolution of the steps movement (a window appears with one of the three values of the discreteness of moving the shelves: 1 °C, 0.5 °C, 0.1 °C).

❖ **Creating a new amplification program without using the Amplification program template**

To create a new amplification program without using a template, procced the following steps:

1. Choose a **Create new program** command. After that, an **Amplification program editor window and an Amplification program template window will be displayed**.
2. Press the button **Cancel** in the **Amplification program template window**.
3. Enter the name of the amplification program in the **Program title** field.
4. Fill in the **Description** field if necessary.
5. Fill in the table with the amplification program, similar to creating an amplification program using a template.

Note: The table with the amplification program can be filled in either using the edit buttons to the right of the table or by the keyboard.

❖ **Filling in the amplification program table using the keyboard:**

To fill in the table of the amplification program using the keyboard, it is necessary to fill in the fields of temperature and time (minutes, seconds). Entering each new value completed by pressing the <Enter> key (Fig.35).

№ block	Temperature °C	min	sec	Number of cycles	📷
1	94.0	0	30	1	

Fig.35 Example of filling temperature and time fields

After introduction of all parameters (temperature, minutes and seconds) of the current line, a new line of the same block with empty fields for input appears automatically (Fig.36).

№ block	Temperature °C	min	sec	Number of cycles	📷
1	94.0	0	30	1	
		0	0		

Fig.36 Example of adding a line

To complete the block, press <Enter> without entering the temperature and time in the new line. After that, the editing of the value of the number of cycles in the block becomes active. Pressing the <Enter> key entering a new value for "Number of cycles" finishes and jumps to enter a new block (Fig.37).

№ block	Temperature °C	min	sec	Number of cycles	📷
1	94.0	0	30	1	
	92.0	0	10	30	
2	62.0	0	10		✔
	72.0	0	10		
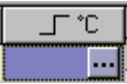
Fig.37 Example of entering the number of cycles

Next, the type of each block shall be specified, enable optical measurements and, if necessary, indicate the temperature change on a matrix, increment on time and temperature (see related topics).

- **Temperature gradient across the matrix (for DTprime only)**.

Using temperature gradient allows entering horizontal or vertical temperature gradient on the matrix if the device is equipped with a solid matrix, or independently to adjust the temperature of each of the six blocks of the matrix (4 x 4), if the device is equipped with pseudo blocks. The maximum value of the gradient on the matrix or the temperature difference between the different zones is ± 10 °C. Thus, using a temperature difference (when setting different temperatures in blocks), six different temperature profiles can be used with one RUN, which allows performing simultaneous reactions with different temperature parameters. The temperature gradient (smooth horizontal or vertical temperature change in the block) is usually used in scientific applications to optimize temperature profiles.

To indicate the temperature drop across the matrix, it is necessary to:

1. Enable option ☑ Temperature gradient over the plate   in the **Amplification program editor** window.

2. Open the window in the corresponding row of the table with the amplification program using the button ⬚⬚⬚. **Use temperature gradient** (Fig.38) (example for DTprime).

3. Select a gradient type ⊙ Vertical   or ⊙ Horizontal   (only for DTprime, for DTlite it is automatically proposed **to use temperature gradient**).
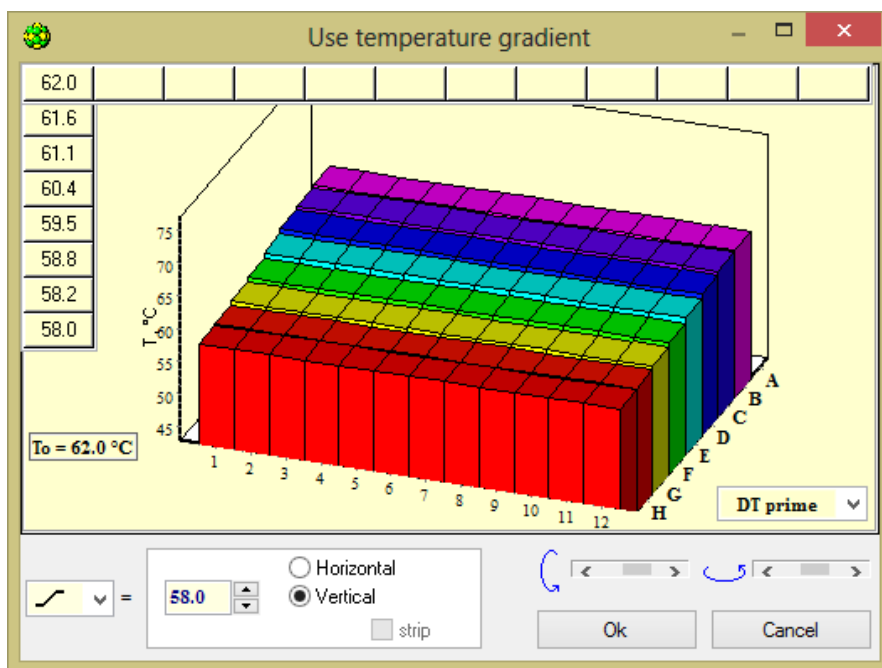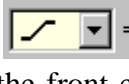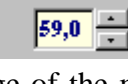
Fig.38 **Using temperature gradient** (Vertical gradient)

4. To enter a gradient in the ⬚⬚ = 59,0 ⬚ windows select a gradient symbol ⬚⬚ and enter the temperature of the front edge of the matrix when selecting a vertical gradient, or the right edge of the matrix when selecting a horizontal gradient (Fig.39, 40).
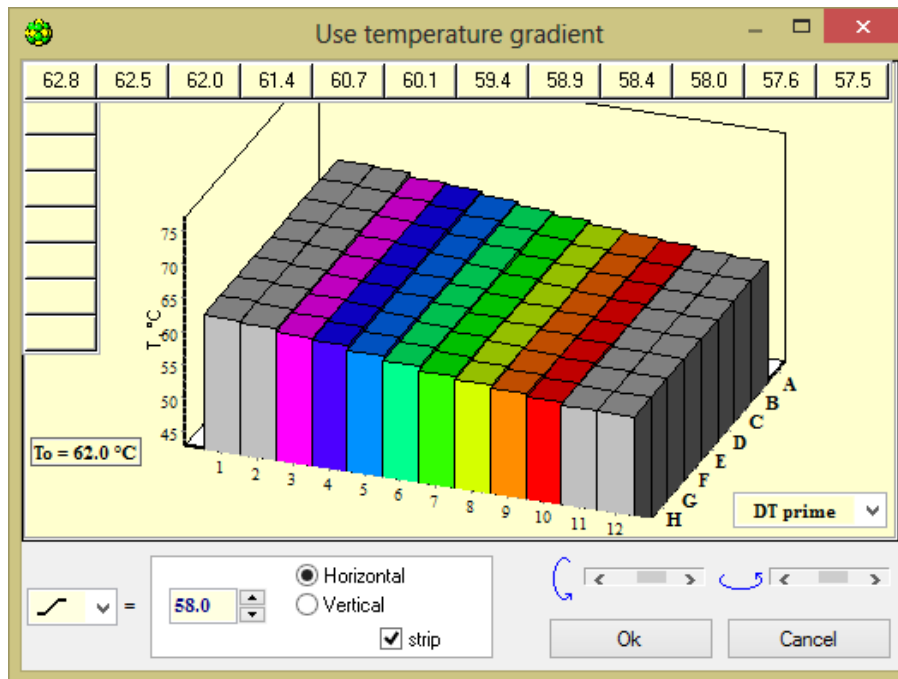
Fig.39 Horizontal gradient with the option "strip"

If you select horizontal gradient, the option ☑ strip can be set, using in this case the field of the 8x8 matrix with improved linearity of the gradient (the colored area on Fig.39).
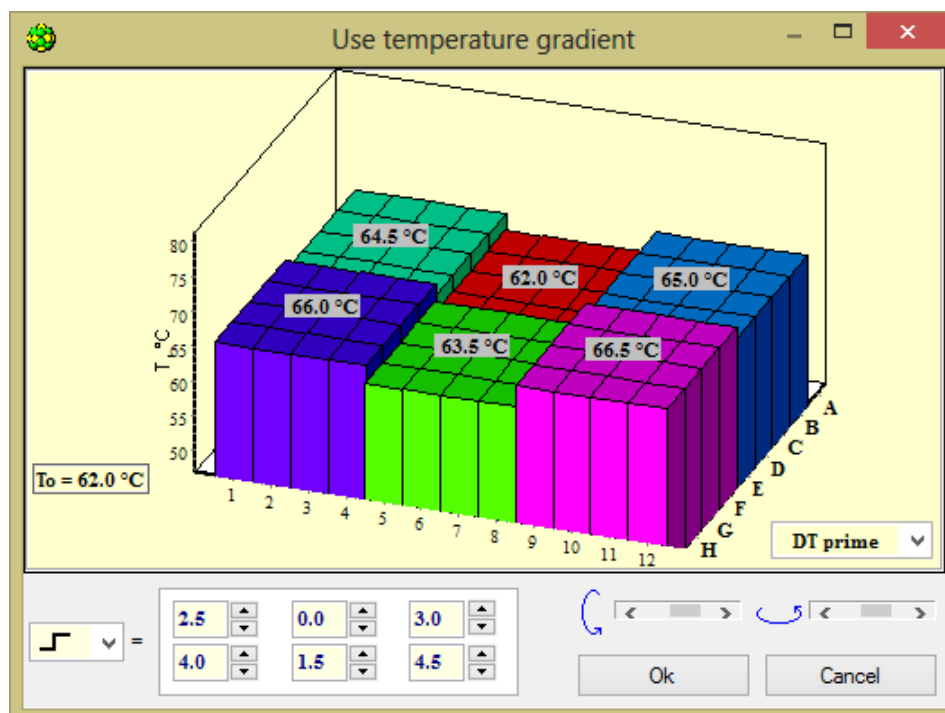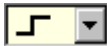
Fig.40 The temperature drop across the blocks for DTprime (only for devices with pseudo blocks)

To enter the temperature difference in blocks in the following windows:

select the symbol of the difference ⌐▼ and enter its value with respect to the shelf temperature for each pseudo block of the matrix (Fig.40).

5. Click **OK** to complete the entry, otherwise click **Cancel**.
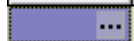
▪ **Increment by time**
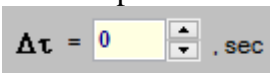
Using the increment by time allows changing a **duration of** the given temperature shelf for each subsequent cycle by the value of a given increment.

To set the increment by time:

1. Enable option ☑ Time increment in the **Amplification program editor** window.
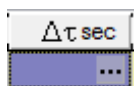
2. Using the [Δτ sec ⋯] button open the window in the corresponding row of the table with the amplification program **Use increment** (Fig. 41).

3. Enable option **Use time increment** (the input field of the increment in time becomes active Δτ = 0 ⇅ , sec).

4. Enter the increment by time.

5. Click **OK** to complete the entry, otherwise click **Cancel**.

In the course of editing the increment value, the duration of temperature shelf is automatically recalculated for each subsequent cycle and it is displayed graphically as a dependency from the cycle number.
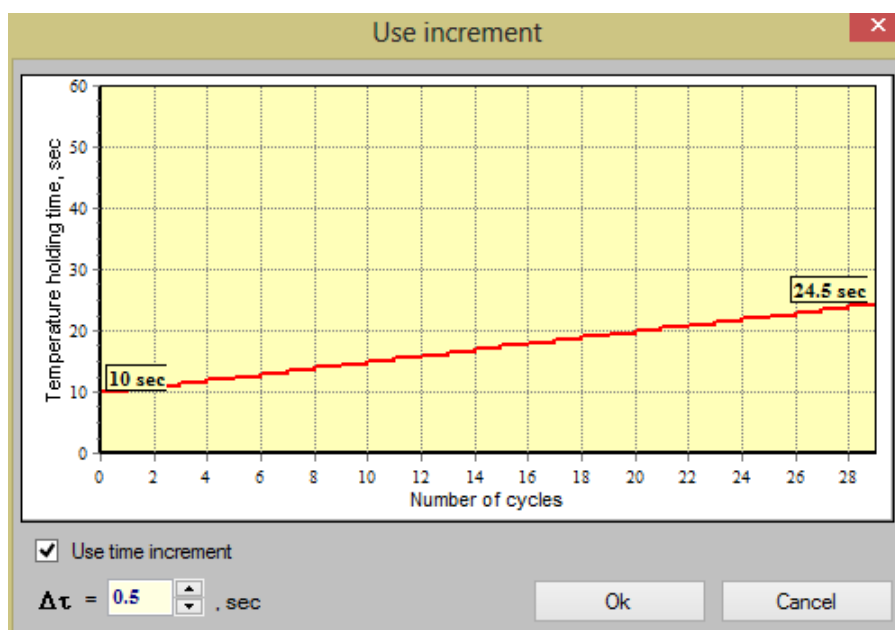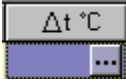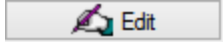

Fig.41 Increment in time

▪ **The temperature increment for the "Cycle" block**

Use the temperature increment to create a "Cycle" block, that allows changing the **value** of this matrix temperature to perform the temperature shelves for each subsequent cycle by the value of the specified increment.

To indicate the temperature increment:

1. Enable option ☑ Temperature increment   in the **Amplification program editor** window.

2. Using the button [Δt °C ...] open the window in the corresponding row of the table with the amplification program **Use increment** (Fig.42) to edit the temperature increment.

3. Enable option **Use temperature increment**.

4. Enter the value of the increment by temperature Δt = [0,40] . °C or the temperature shelf for the final block cycle (the increment will be calculated automatically) Tend = [51.8] . °C.

5. Click **OK** to complete the entry, otherwise click **Cancel**.

In the course of editing the increment value, the temperature of the shelf is automatically recalculated for each subsequent cycle and it is displayed graphically as a dependency from the cycle number.
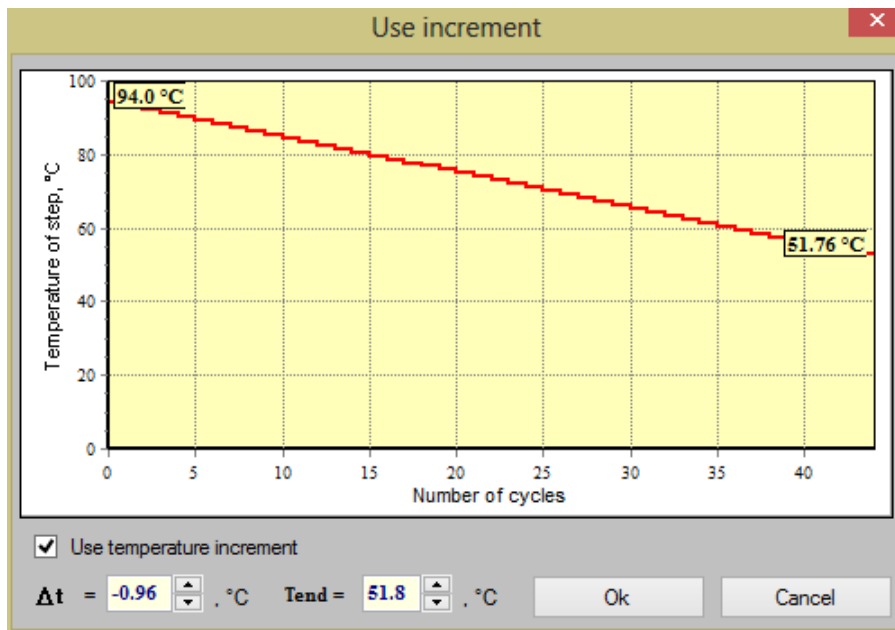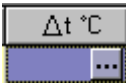
Fig.42 The temperature increment for the "Cycle" block

▪ **The temperature increment for the "Melting curve" block**

Using the temperature increment when creating the "Melting curve" block allows editing the initial parameters of this block.

For that, procced the following:

1. Enable option ☑ Temperature increment   in the **Amplification program editor** window.

2. Using the button [Δt °C ...] open the window in the corresponding row of the table with the amplification program **Melting curve** (Fig.43) to edit the initial parameters of this block.

3. Set three of the four parameters in the window **Melting curve** (for example, initial temperature ☑ [90.0] To, °C , temperature increment ☑ [-0.50] dT, °C , and the number of cycles ☑ [100]). The fourth parameter will be calculated automatically.

4. Click [Apply] to complete the entry, otherwise click [Cancel].

In the course of editing the parameters of the Melting curve window, the temperature of the shelf is automatically recalculated for each subsequent cycle and it is displayed graphically as a dependency from the cycle number.
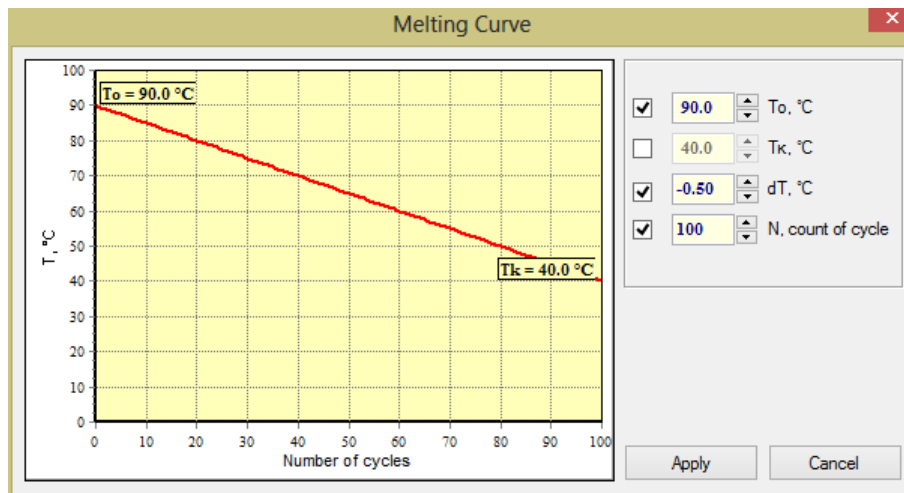
Fig.43 The temperature increment for the "Melting curve" block

4.1.6.3 Editing the amplification program

Edit existing amplification programs at various stages of the **RealTime_PCR software**:

1. When creating and editing tests:

   – open **Test** window;

   – press the button **Edit** in section **Amplification program**;

2. Before starting the amplification program:

   – select a tab **Start run** in the **RealTime_PCR** window;

   – press the button **Edit** ;

3. When working in Amplification program editor:

   – select item of the Menu of **Amplification programs editor window:** - open the program from the hard disk or a data storage device.

To edit the amplification program, complete the following actions:

1. Choose the **Edit program** command, then **Amplification program editor window** will be displayed.

2. Edit the amplification program, similar to creating an amplification program using a template.

4.1.6.4 Saving the amplification program

To save the amplification program:

1. Press the button ![Ok button] (to save the amplification program and exit) the **Amplification program editor**.

2. In the dialog box that appears (Fig.44), enter the name of the created amplification program in the field **File name**, using the Latin letters only.

3. Using a **Folder** field, select a folder on the hard disk or a data storage device to save it (by default the software will offer the operator's working directory).

4. Press the button **Save**. The amplification program will be saved to a file with *.rta extension.
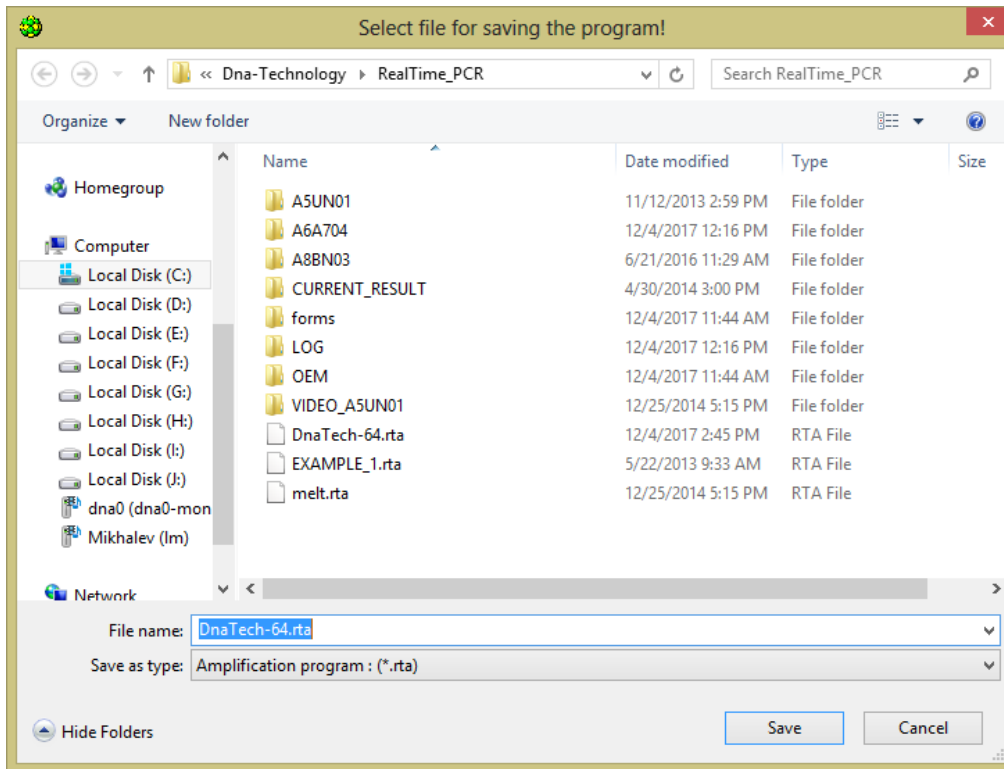
Fig.44 Saving the amplification program

4.2 Creating a plate protocol.

4.2.1 General information about the plate protocol.

Before the amplification, it is necessary to fill in the plate protocol (Tab **Protocol** in the window **RealTime_PCR**).

The plate protocol includes the following parts:

1. Information about the analysis:

 – the identificator (description) of each tube;

 – type of each tube;

 – the number of duplicates of each sample;

 – concentration of calibration samples (for quantitative studies);

 – fluorophore used (fluorescent dye) and its purpose;

 – amplification program;

 – the location of the tubes in the thermal block of the device;

2. Additional information:

– plate protocol number;

– operator name;

– date of the analysis.

The plate protocol data is displayed in a table, each line of which contains information about a single tube (Fig.45).
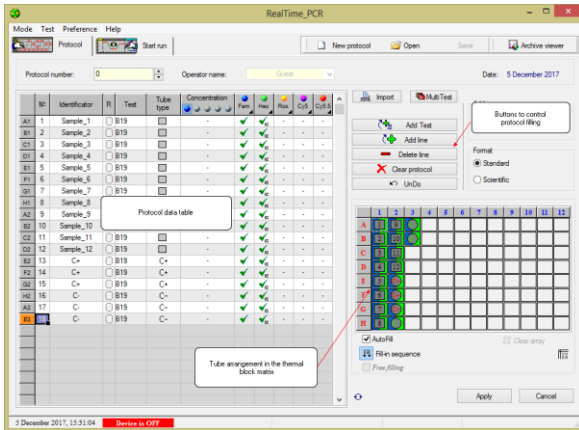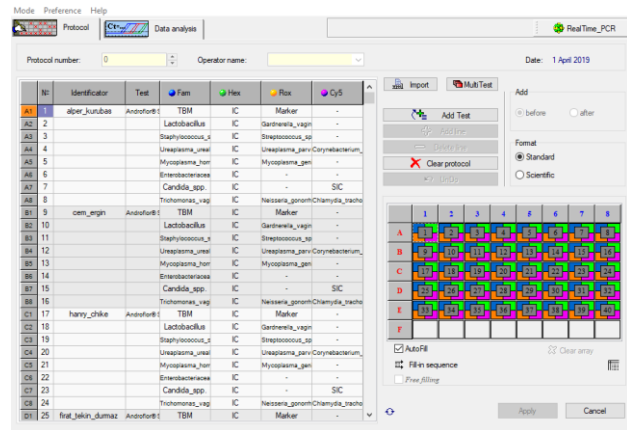
(For DTprime)   (For DTlite)

Fig.45 "RealTime_PCR" window, plate protocol tab

The location of the tubes in the thermal unit of the device is schematically displayed in the lower right part of the "RealTime_PCR" window (Fig.45).

Editing the plate protocol is performed using the buttons located to the right of the table (Fig.46).
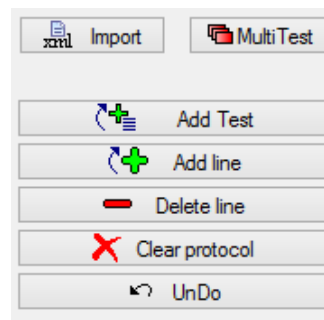
Fig.46 Buttons for editing protocol

The plate protocol can be filled in the following ways:

1. Using the option **Test** with the saved standard parameters of the analysis.

   Tests (using options **Create/edit** or **Copy group of tests**) are available to the Operator who created them and may be accessed by the button **Add test**.

2. By filling the plate protocol manually line by line.

3. Use the previously saved plate protocols.
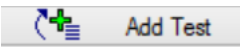
4.2.2 Import protocol data from the LIS (Import button ![Import](xml Import)).**Create a plate protocol using the Test option**

To create a plate protocol using the Test option, perform the following actions:

1. Log into the RealTime_PCR software under one of the operators, using the **Device operation** mode and select the device to connect.

2. If necessary, specify the plate protocol number in the **Protocol number** field

   Protocol number: [0]
   .
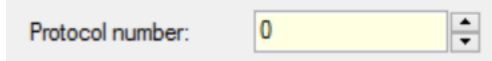
3. Click on the button **Add test** ![Add Test] of the **Protocol tab.** The window **Add test is** opened (Fig.47).

4. From the drop-down list of **Test** (located to the right) select the test type: **Single** (for single-tube tests), **Complex** (for multi-tube tests) or **All tests**.

   ALL Test
   > ALL Test
   > Single Test
   > Complex Test
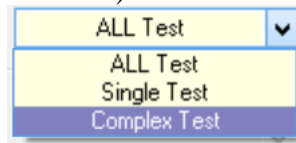
5. The drop-down list of Test located to the left will display the tests of selected type.

6. Select a test name from the drop-down list of **Test**. The parameters of the selected test are displayed in the window.

7. Specify the number of test samples and the number of replicates of each sample in the section **Samples**.

8. Specify the number of tubes with control samples in the section **Controls**.
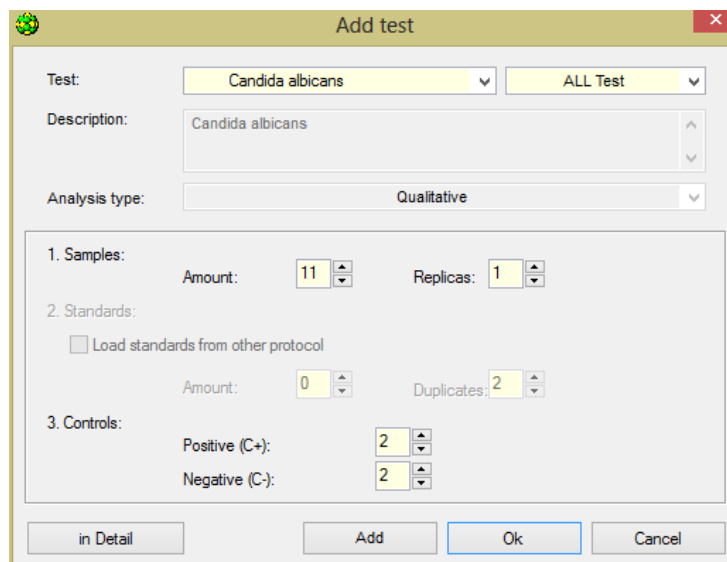
Fig.47 "Add test" window

9. When carrying out the quantitative analysis, specify the number of calibration samples and the number of repetitions of each sample in the section **Standards**.

   Note: During the analysis, it is possible to use "standards" from another RUN (for more details, see section **Additional standards**), to enable it proceed the following:

   – enable option **Load standards from other protocol**;

- using the **Browse** button, open the file selection window with additional standards;

- select the corresponded RUN.

The path to the selected file will be displayed in the **Standards** option.
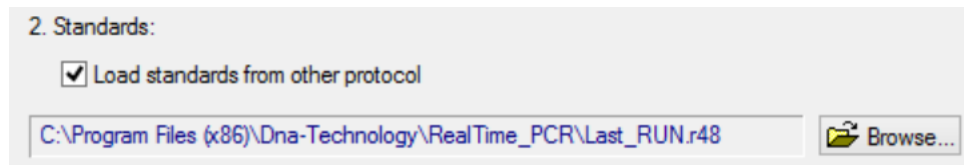
Attention! The obligatory condition for the use of additional standards is the identity of the plate protocols for the amplification program, as well as the absence of changes in the list of tests of this Operator.
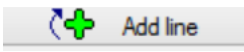
10. To save data and return to the **RealTime_PCR** window click the button **OK**, to return to the **RealTime_PCR** window without saving the data, press the button **Cancel**.

11. When using the procedure **Add test**, the fields of the plate protocol are filled in automatically.

12. Only tests of the same type (matching by type of analysis and amplification program) can be added in one plate protocol. Additional editing of the plate protocol is possible (change of identificators names, concentration, standards, etc.) manually.

4.2.3 The manual line-by-line plate protocol filling

In this option all information in the plate protocol fields is manually entered.

Note: With a line-by-line plate protocol creation, it is impossible to change the type of data analysis, a qualitative analysis type will automatically be used.

To fill the plate protocol manually, perform the following actions:

1. Log into the RealTime_PCR software under one of the operators, using the **Device operation** mode and select the device to connect.

2. If necessary, specify the plate protocol number in the **Protocol number** field.

3. To add a new line (description of the new tube), press the button **Add line** .

4. The following data in the line can be edited:

- *No.* (tube number). Filled automatically when adding a new line, if necessary, the data can be edited;

- *Identificator* (short designation of the sample) - an arbitrary description of the tube such as patient's name, sample number, etc;

- *R* (adding duplicates). Each time pressing a button in the corresponding row, a linked line is created in the R field with an identical description of the tube (duplicate). Creating duplicates is possible after entering all the information about the tube. To change information about the block of duplicates, the "head line" of the block of duplicates has to be edited;

– **Tube type.** The following types have to be selected from the drop-down list: Sample, Standard, Control + or Control - ;
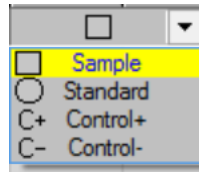
– **Concentration.** Filled with the **Standard** type of tube. It is necessary to indicate the concentration of the calibration sample;

– **Fam, Hex, Rox, Cy5 and Cy5.5** (*for a 5-channel device*) - choose from the drop-down list the purpose of the fluorophore (specific target, IC, not detected).
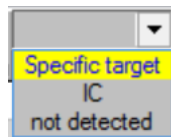
The field **Test** remains unfilled.

Note: When preparing to RUN in this way, the type of analysis is Qualitative.

Once the amplification program is completed, the following changes can be made to the plate protocol: the type of analysis, the values of the calibrators, the type of tubes, duplicates, identificators.

Attention! Do not fill a plate protocol without using the **Test** procedure (using options **Create/edit** or **Copy group of tests**) since the **Analysis parameters** window (see section 8 of this manual) will not be active, and the optical calibration coefficients of the device will not be used.

4.2.4 Location of tubes in the thermal block matrix

After filling in the plate protocol fields with the tubes' data, it is necessary to specify the location of the tubes in the thermal block of the device. Tubes can be placed in the matrix automatically or manually.

When filling the plate protocol by any of the above methods, the tubes are automatically placed in the thermal block matrix.

Using the button ⇉ Fill-in sequence   choose the order of filling: by rows, or by columns (Fig.48).
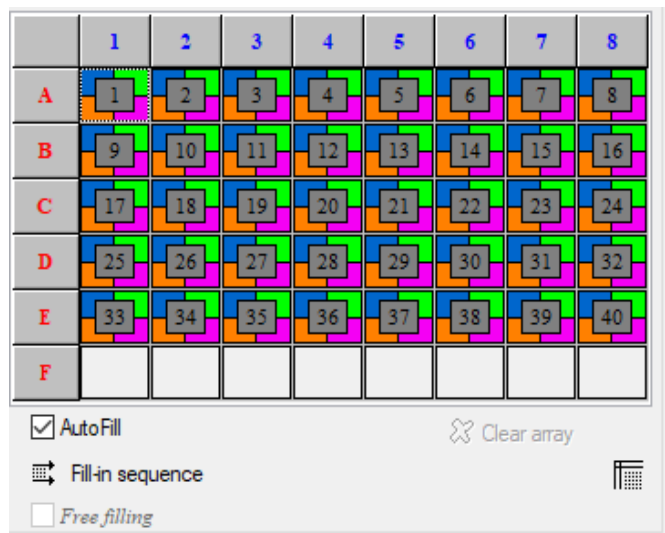
(For DTprime)   (For DTlite)

Fig.48 Schematic arrangement of test tubes in the thermal unit matrix

Tubes can be placed manually in a free order. To do this, disable the ☐ AutoFill mode, tick the ✓ Free filling mode, clear the matrix field using the button ✗ Clear array , place all the tubes in the matrix using the mouse cursor.

Button ▥ is intended for displaying a graphic image of a matrix window. This image can be printed or saved to a file.

For different types of device, the schematic arrangement of the tubes will be different. This depends on matrix capacity of the device.

To edit and start the amplification program, press the button **Apply**.

After clicking the button **Apply** the software automatically switches to the **Start run**.

4.2.5 Start the amplification program

Start the amplification program on the tab **Start run** of the **RealTime_PCR** window after creating a plate protocol (see "Creating a plate protocol").

By default, the tab displays the program entered by the operator when **creating/editing test**. When creating a Test using **ini-files**, the necessary program is loaded automatically (Fig 49).

When creating a plate protocol using the option **Add test**, it is not necessary to edit parameters in this window.

If the plate protocol was created manually line-by-line, in this window select an amplification program from a previously created amplification, edit one of the previously created, or create a new one and specify the volume of working mixture in tubes.
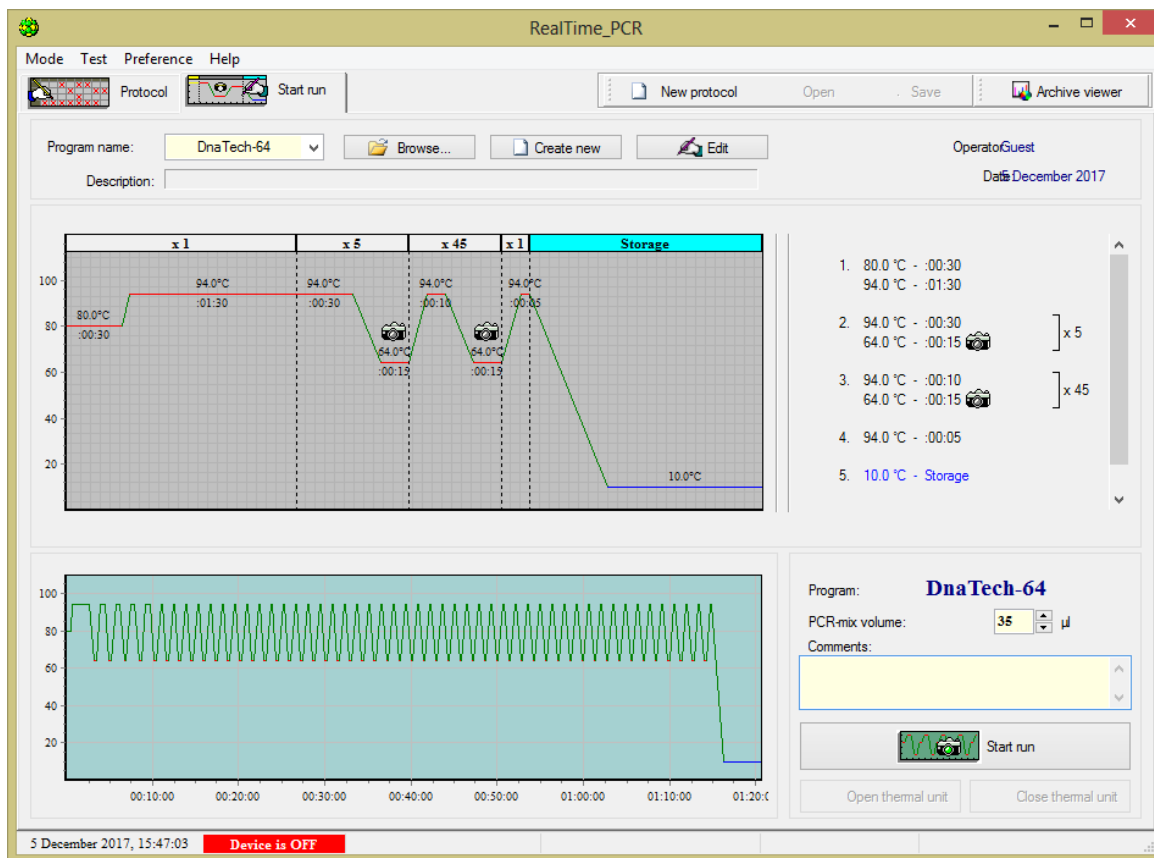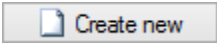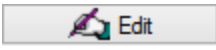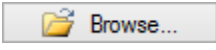
Fig.49 "Start RUN " tab in the "RealTime_PCR" window

- create a new amplification program (button [Create new]);

- edit the current amplification program (button [Edit]);

- select an amplification program from the drop-down list [Program name: DnaTech-64] (the list shows the last 10 programs used);

- upload the amplification program from hard disk or any other source (button [Browse...]).
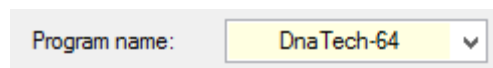
After selecting an amplification program, it will be displayed in various representations: a graphic image, a text representation and a temperature profile of the amplification program.

1. If necessary, edit **PCR-mix volume** in a tube.

2. Fill in the **Comments** field, if necessary.

3. Press the button **Open thermal unit** (block) for its initialization**.**

4. Press the button **Close thermal unit** (block).

5. Press the button **Start run**.

After the thermal block is in working position a dialog box appears on the screen of the display that prompts to specify the name of the RUN file in which the results of optical measurements will be stored and a place to save it (by default it is a working directory of operator) (Fig.50).

6. Select the directory to save the RUN file (in case it differs from the default directory).

7. **File name:** enter the name (agree with the unique file name proposed by software or create another file name).

8. **File type:** the data saving format will be specified with the following extensions, which are corresponded to type of the device: r48,*.r96, *.384.
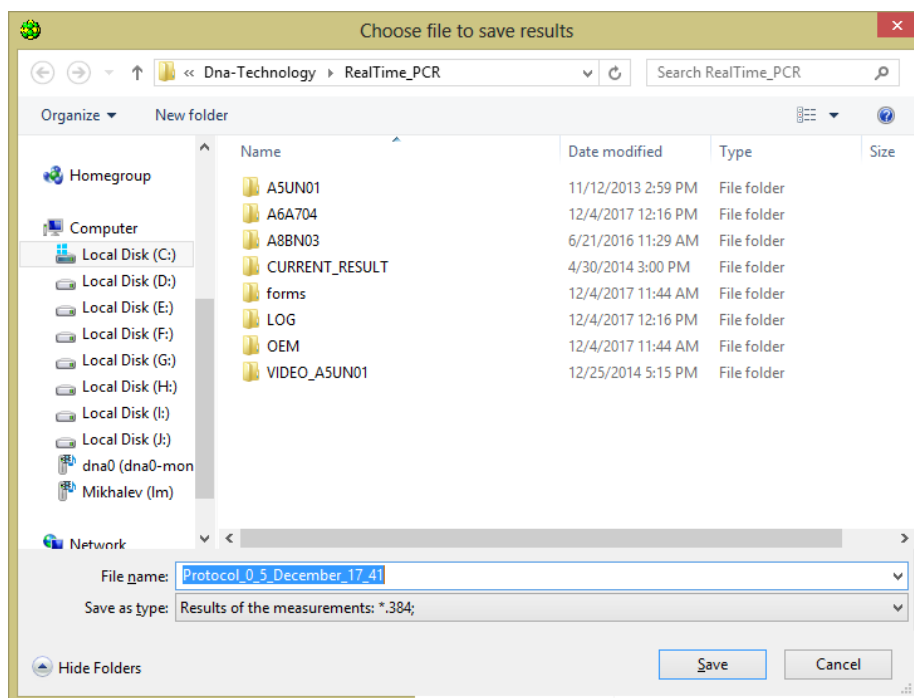
Fig.50 Select a file to save the results of optical measurements

After clicking on the Save button, the RealTime_PCR software processes into the mode of executing the amplification program while saving the results of optical measurements in the RUN file.

In the status line of the software, one of the following states represents the device status:

Device is OFF – the device is switched off or not connected to the computer.

Device is ON – the device is switched on, but not ready for operation (warming up the device for at least 5 minutes).

Device is ON – the device is switched on and ready for use.

Note! The activation of the amplification program is possible with the status of the device – "Device is ON" (green or yellow indicator in the status line of the software). In the case of a status of the device – "Device is OFF" (red indicator), a message will be displayed that the device is not connected (switched off).

Attention! If you pressed the Start RUN button during warm-up (yellow indicator is in the status line **Device is ON**), in the **Temperature profile** window a flashing message will be displayed on the continuation of the warm-up process of the device (Fig.51):
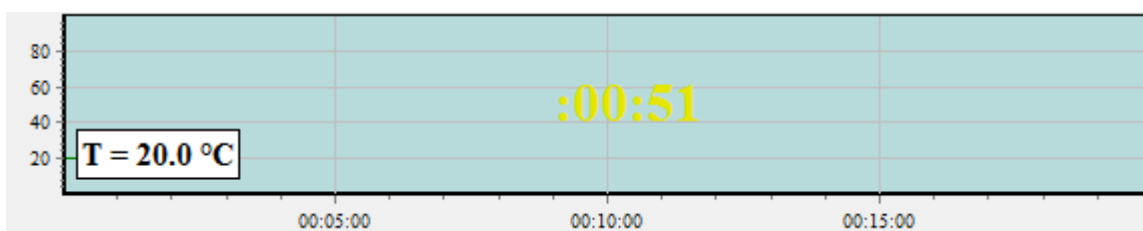
Fig.51 Message about the remaining warm-up time

A similar message will be displayed on the device's display. After the end of warm-up, the device will automatically switch to the mode of the amplification program execution.

4.2.6 Use the option Switch off the device after the RUN

If there is a need for autonomous operation of the device with its automatic shutdown (transition to sleep mode) after the completion of the amplification program, perform the following:

1. In the **Running** window enable the following option:
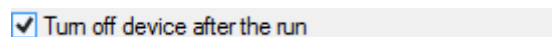
2. Close the software by pressing in the upper right corner of its window.
3. Upon completion of the amplification program, the device processes into a sleep mode, and indicates by the blinking LED on the OSD menu button (only for DTprime device).

Attention! The device will switch into a sleep mode only if the option **Turn off device after the run** is activated and the amplification program performed therein ends in a **Holding** mode.

In case of closing the RealTime_PCR software (during the autonomous work of the device), the computer remained turned on, the file with the results of optical measurements will be saved in the chosen directory and the operator will be available to view it after the amplification program is completed.

Note! In case of closing the RealTime_PCR software (during the autonomous work of the device) the computer was turned off, the file with the results of optical measurements will not be saved in the chosen directory. The RUN file can be downloaded to the computer via the option **Last run in device** in the menu **Preferences** of the **RealTime_PCR** software window.

4.2.7 Running the amplification program

If the amplification program starts successfully, the tab **Start run** of **RealTime_PCR** window is replaced by a tab **Running** (Fig.53). After starting the amplification program, the device does not require user intervention. If necessary, during the program execution, the operator can change the display of the current processes and also change the amplification program (only for advanced users).

After the amplification program is completed, the software automatically switches to the data analysis viewing mode with the results of optical measurements.
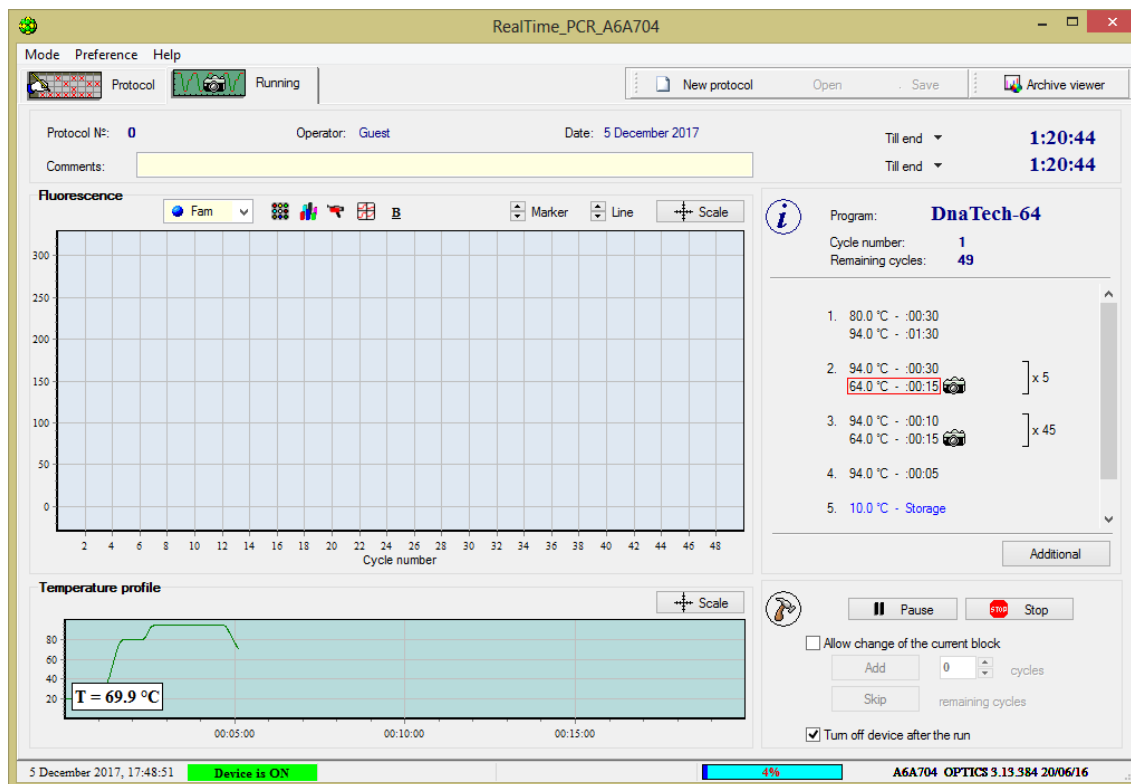
Fig.53 The **Running** tab of the RealTime_PCR window

The top part of the tab displays general information about the current RUN:

-- protocol number;

-- operator name;

-- date;

-- comments on the RUN, which, if necessary, can be supplemented in the process of program execution;

-- two fields in the right corner of the window, where the following time parameters for displaying can be selected from the drop-down list:
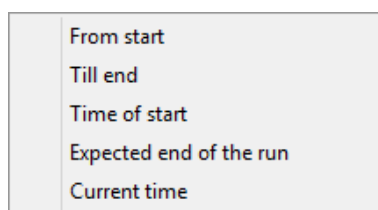
In the central part of the tab, a graph of fluorescence values is displayed: the DNA accumulation curves in each tube in the coordinates "Fluorescence level – amplification cycle number".

The choice of the fluorescence channel for displaying fluorescence values on the graph is performed using the following drop-down list:

45
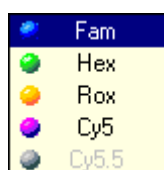
To control the display mode of the graph and detailed analysis of the data, the following elements can be used:

Marker – buttons for changing the size of markers;

Line – buttons to increase/decrease the thickness of lines;

Scale – button to display the dialog box for changing the scale of the axes (Fig.54):
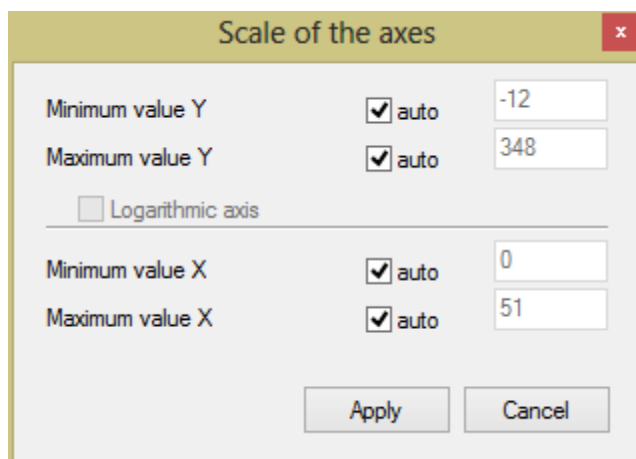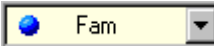
Fig.54 Adjusting axis scale

– **Location and color of the tubes** button, by this option the color of the tubes, as well as the view of their type and numbering can be changed.

– **Distribution of optical measurements over the plate** button shows a graphical representation of the current values of optical measurements on a given cycle in three-dimensional coordinates (Fig.55).

In the **Distribution of optical measurements over the plate** window you can see the value of the selected fluorescence channel **Fam** for each cycle **18**.

Using the arrow buttons located at the bottom of the window, zoom in and out in a horizontal or vertical plane.

The window can be resized by fixing the mouse cursor on the border of the window until the double arrow appears, then moving the border using the mouse.
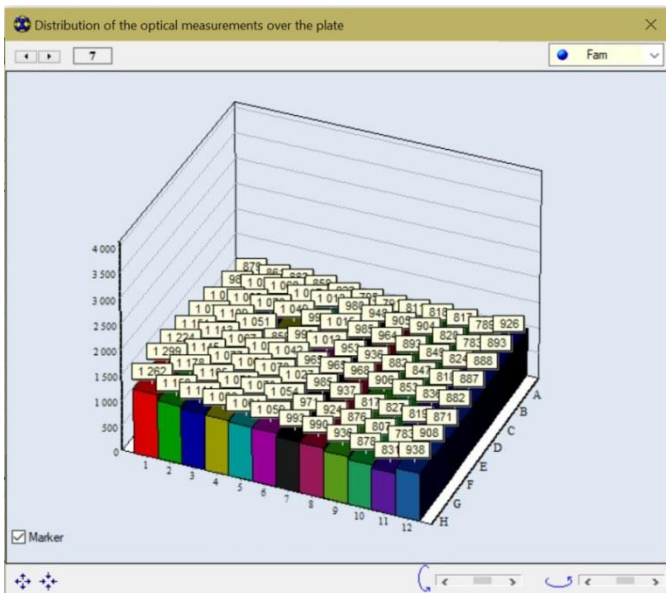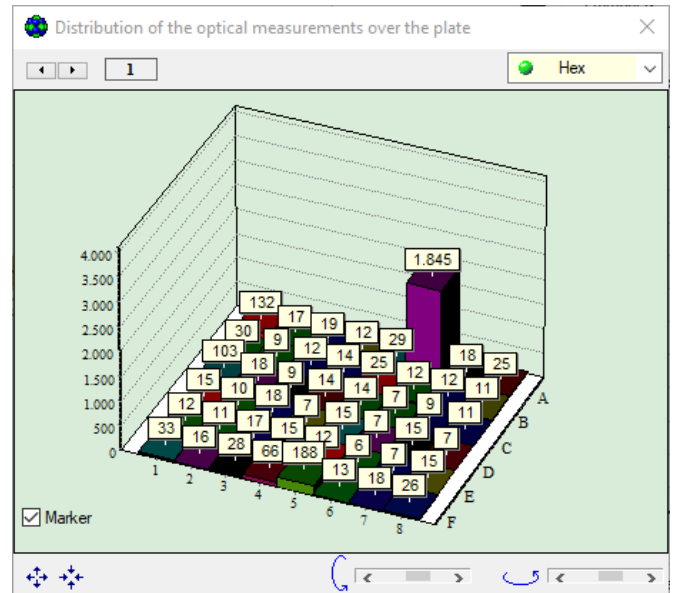
(For DTprime)　　　　　　　　　　　　(For DTlite)

Fig.55 **Distribution of optical measurements over the plate** window

– **Current image** button is intended to display the window with the image of the matrix field with tubes on this cycle for a given measurement (Fig.56).

B – button is intended for switching on/off of the calculation mode of the basic (background) component for each tube by the "first" cycles of the amplification program. All measurements are output after deduction of this base component.
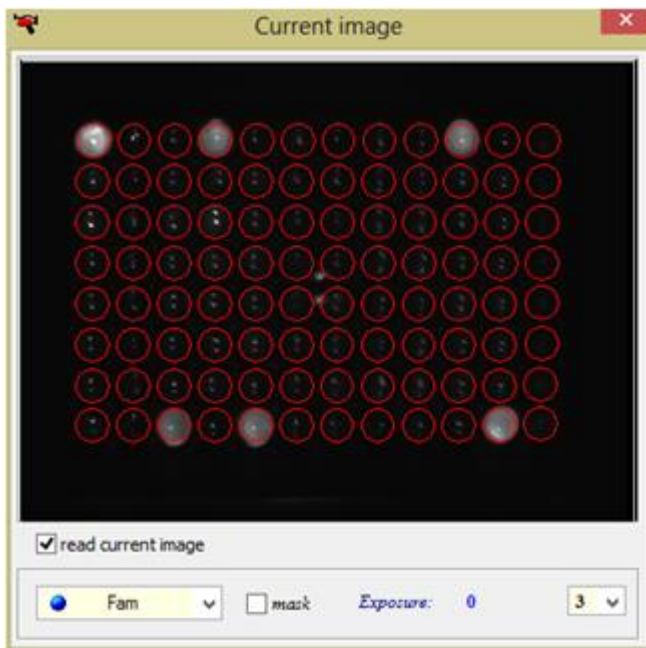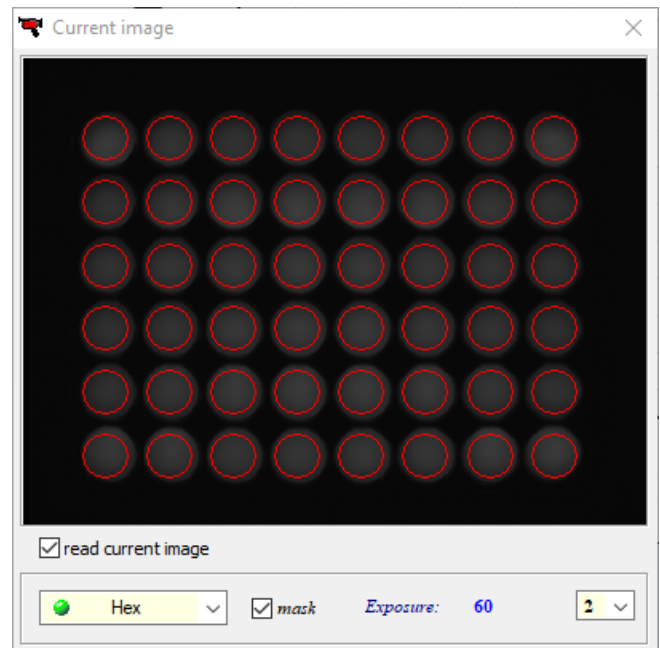
(For DTprime)　　　　　　　　　　　　(For DTlite)

Fig.56 «Current image» window

Note (for advanced users): To specify the boundaries of the first cycles, select item **Options**... in the menu **Preferences** of the **RealTime_PCR** window. In the appeared **Options** window select a tab **Optical data acquisition**, enable the option **Consider background input starting from** and indicate the boundaries of the cycles in the corresponding windows (Fig.57).

Fig.57 Specifying the boundaries of the first cycles

⊞ – **Change the representation of optical data** button is intended to display a multi-window view of the curves (each kinetic curve is displayed in its own window See (Fig.58).
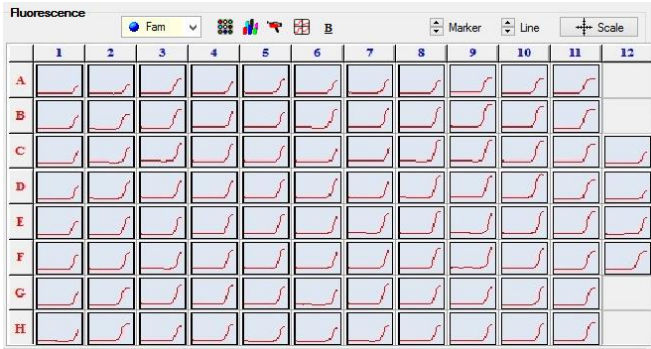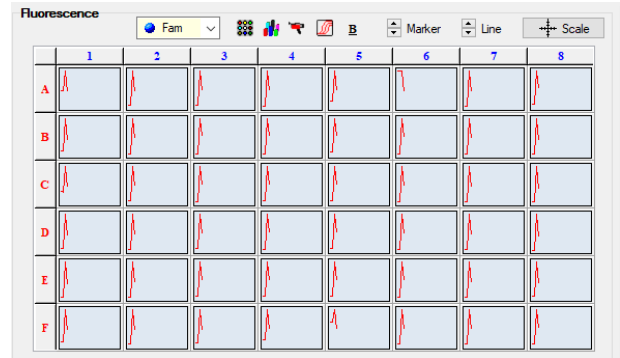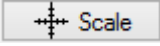
(For DTprime)   (For DTlite)

Fig.58 Multi-window view of curves

At the bottom of the Running tab, the **Temperature profile** is shown (diagram of the matrix temperature versus the time of the amplification program). Temperature shelves with optical measurements are highlighted in red, the moments of optical measurements are displayed by flashing 📷 the icon on the diagram.

On the right above the field with the diagram, there is the button ⊹ Scale for displaying the dialog box for changing the scale of the axes.

The area indicated by the symbol 𝒊 shows the progress of the amplification program and contains the following information (Fig.59) :

- – the name of the amplification program;
- – number of the current cycle;
- – the number of cycles remaining until the end of the program;
- – parameters of the amplification program.
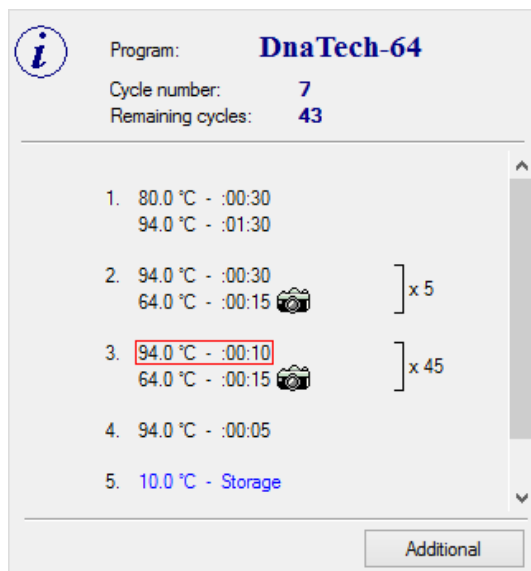
Fig.59 Information on the progress of the amplification program

In the area indicated by the symbol ⟨symbol⟩, there are buttons for controlling the progress of the program:
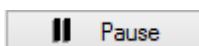
‖ Pause – enable/disable Pause mode (pause the RUN execution). The pause occurs on the first temperature shelf after switching on this mode. When the pause is switched off, a transition to the next temperature shelf immediately occurs, without waiting for the end of the current shelf, at which stage a pause of the amplification process was occurred.
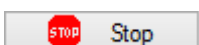
STOP Stop – emergency stop of the RUN execution. **Add** – add the number of cycles in the block entered in the field with the same name.
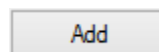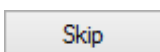
Skip – skip the cycles remaining until the end of the block execution.

Attention! These operations can be performed only with the enabled option ☑ Allow change of the current block
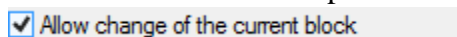
To automatically turn off the device after execution of the amplification program, enable the option ☑ Turn off device after the run .
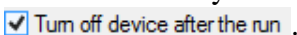
During the execution of the amplification program, the progress bar of the running process is displayed in the status line ▮▮▮ **61%** ▮▮▮ .

During the optical measurements (until the end of the amplification program), if necessary it is possible to come back to the tab **Protocol** and edit the plate protocol for the following parametrs: the number, type, location of the tubes (except for the amplification program). When going back to the tab **Running** after editing the plate protocol, it is requested to save the plate protocol or discard changes.

Note (for advanced users): During the optical measurements, it is possible to change the settings of the measurement and data representation modes. For this, it is necessary to select item **Options...** in the menu **Preferences** of the **RealTime_PCR** window. In the appeared **Options** window select a tab **Optical data acquisition**.

The following parameters can be modified:

- *Show calibrated data* – show the data of the optical measurements taking into account the compensation of the optical uniformity of the matrix;

- *Use DT normalization method* – apply the DT normalization method to calibrate optical measurements;

- **Consider background input starting from** – the background component for each tube is calculated according to the first (indicated) cycles. All measurements are output after deduction of this background component;

- *Auto exposition adjustment* – automatic selection of the exposure value of optical measurements (temporarily unavailable);

- *Number of exposures for one measurement* – the number of measurements (1-4) within the same optical channel: Fam, Hex.

After the amplification program has finished, a window appears on display with a message that the program enters to the **Holding** mode (Fig.60).

In this case, a flashing inscription **Holding**... and a marker with a storage temperature appear on the temperature's graph of the matrix dependence against the time of the amplification program.
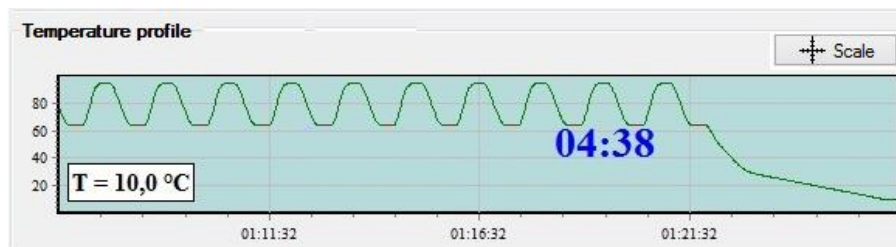
Fig.60 **Holding** mode indication

When the storage temperature is reached, a window appears on the screen indicating that the amplification program has completed its execution and switches to the storage mode (Fig.61).
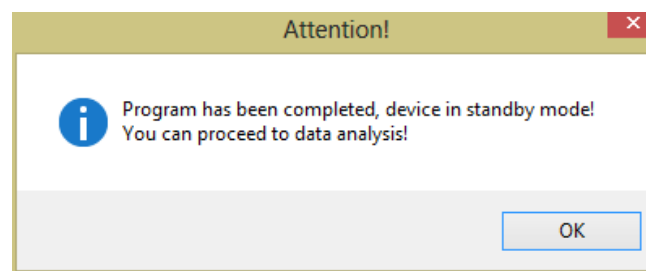
Fig.61 Message about the completion of the amplification process

When **clicking OK** button, the **RealTime_PCR** software switches to the mode **Archive viewer** (tab **Data analysis**).

5 Archive viewer mode

5.1 Mode assignment

Archive viewer mode of the **RealTime_PCR** software is intended for the data analysis of the executed RUN with results of optical measurements and formation of report based on the results of analysis. To open the **Archive** window use one of the following options (Fig.62).

1. Select mode **Archive viewer** when you run the **RealTime_PCR** software.
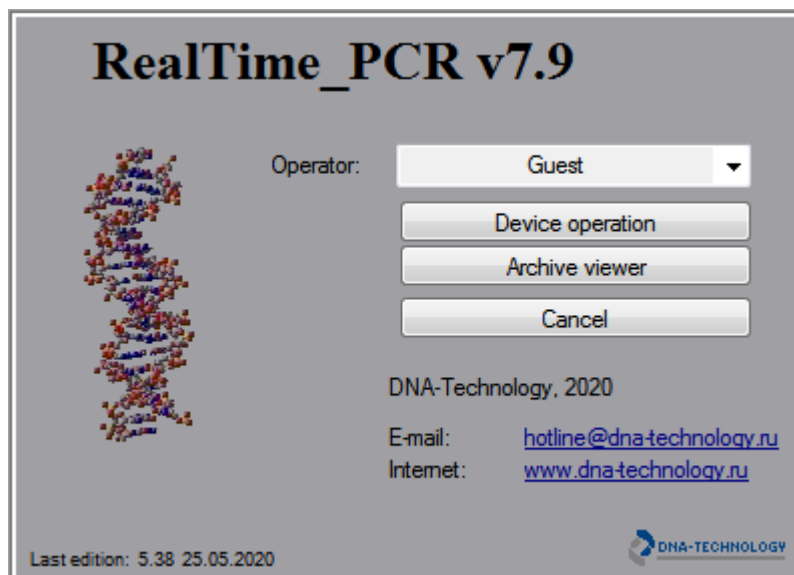
Fig.62 «Program **RealTime_PCR** start» window

2. Select item **Archive viewer** in the menu **Mode** of the **RealTime_PCR** window.

3. Click the button [Archive viewer] of the **RealTime_PCR** window.

4. Double-click any saved RUN file on the computer (file type *.r48, *.r96, *.384). In this case, the RUN file with the results of the optical measurements will be opened immediately (Fig.63).
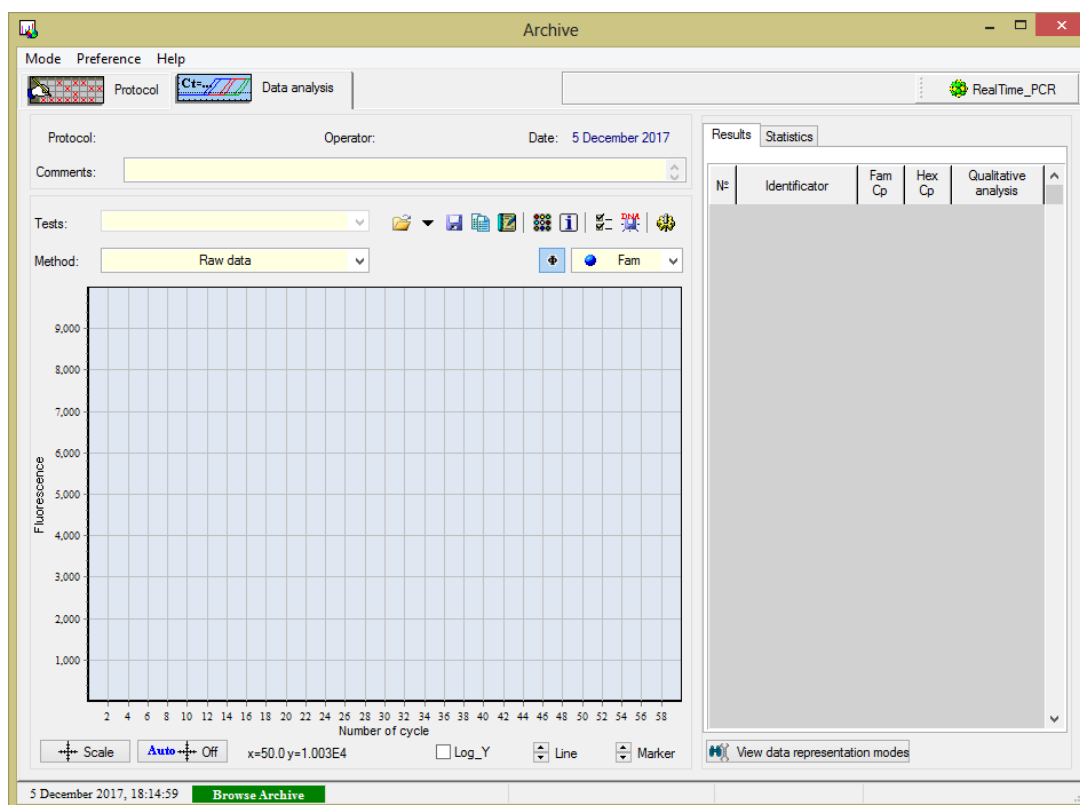
Fig.63 The Archive mode window

5.2 Opening of the RUN file with optical measurements data

After the amplification program is completed, the software automatically switches to the data analysis viewing mode with the results of optical data measurements (Fig.64).

The RUN file with the optical measurement data is stored in the operator folder and can be opened for viewing in the Archive window as well.

RUN file can be opened by several ways:

   - double click to open the RUN file, that is stored on the computer or any other source, with the results of optical measurements (file type *.r48, *.r96, *.384);

   - in the window **"Archive"** click the button 📂 in the **Data analysis** tab and select the necessary RUN file in the **"Open run file"** window.

Note: If for some reason the computer communication with the device was interrupted during the amplification program execution, there is the possibility to download data from the device, that was last Run. To do this, select the item **Last Run in device** in the menu **Preferences** of the **RealTime_PCR window (option is available only in the "Device operation**" mode).
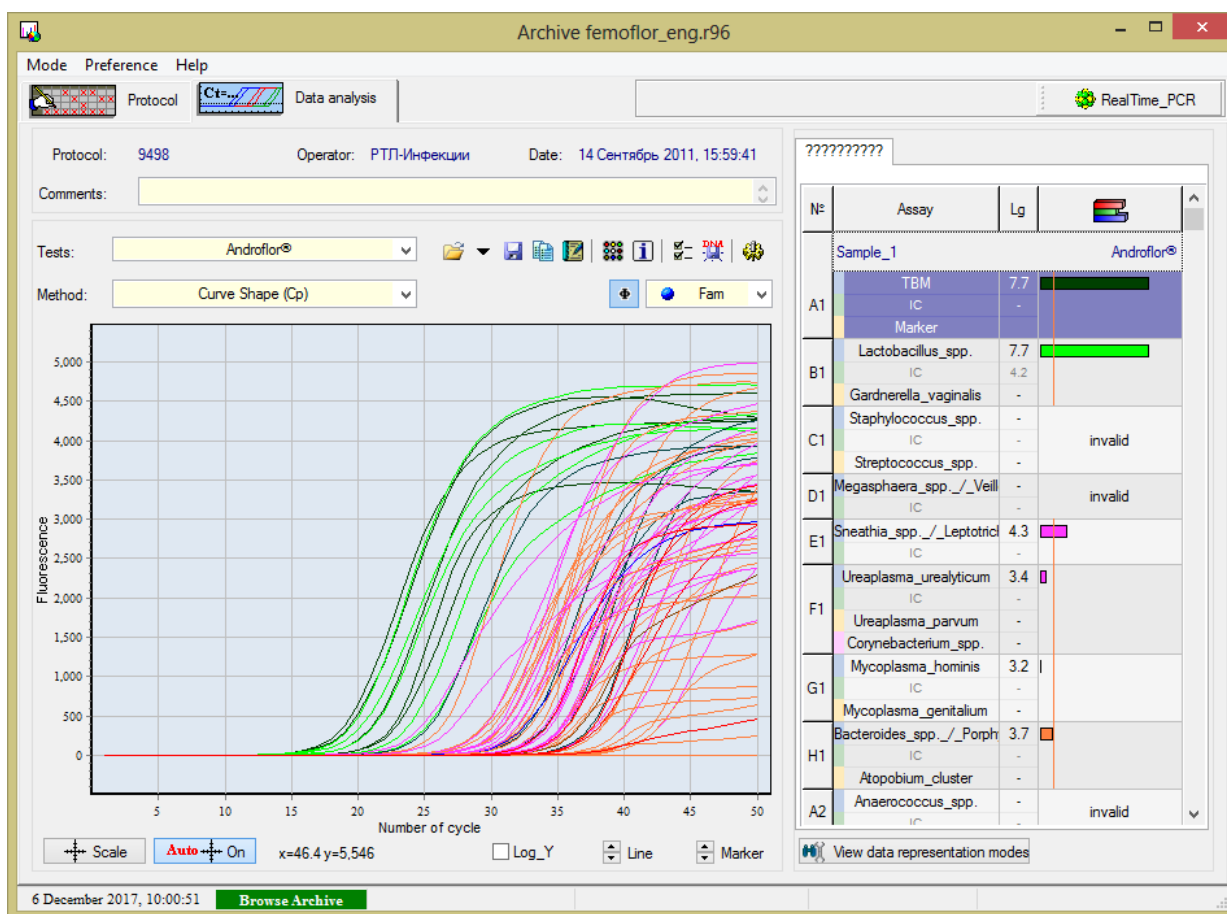
Fig.64 "Data **analysis**" tab of the RUN

5.3   Setting up the analysis parameters

5.3.1 RUN Information

In the **Archive** window the following information about the executed RUN is provided:

1. RUN data (RUN number, operator name, date and time of optical data obtaining, comments to the RUN). The RUN data is displayed at the top of the window.

2. Additional RUN data (the name of the RUN file, the name of the amplification program, parameters of the amplification program, the volume of the reaction mixture in the tube).

For more information about the protocol, click the button 🔳 **Information about amplification program**, which opens the **Information** window. The window contains two tabs:

- **Amplification program** tab contains the name of amplification program, its parameters and volume of the reaction mixture in the tube.
- **General information** tab contains the path to the RUN file, its name, serial number of the device and plate barcode (Fig.65):
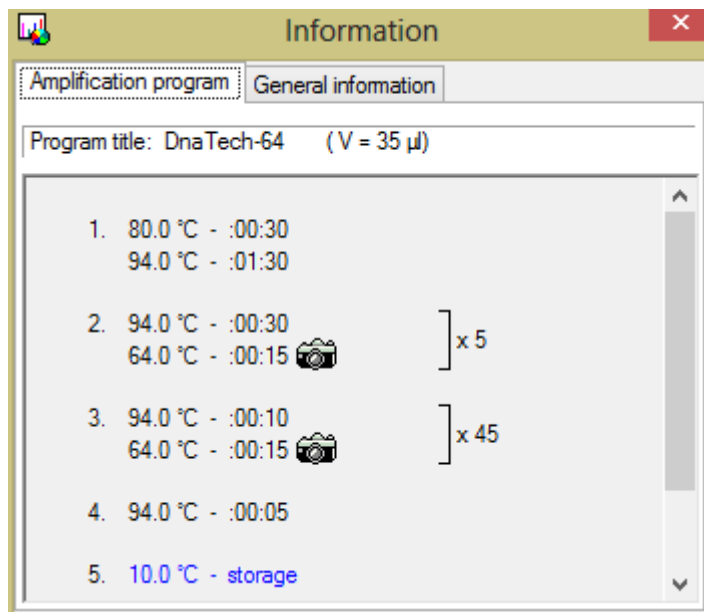
Fig.65 Information about the amplification program

5.3.2 Adjustment of the display and processing the measurement results

Basic toolbar:

, where
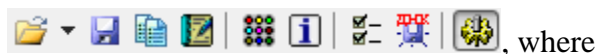
1. 📂 - open the RUN + select from the 10 latest RUNs
2. 💾 - save the RUN
3. 📄 - laboratory report of the PCR analysis
4. 📝 - medical form of automatic report of the PCR analysis
5. ⠿ - location and color of the tubes
6. 🔳 - RUN Information
7. ☑ - change parameters of data analysis
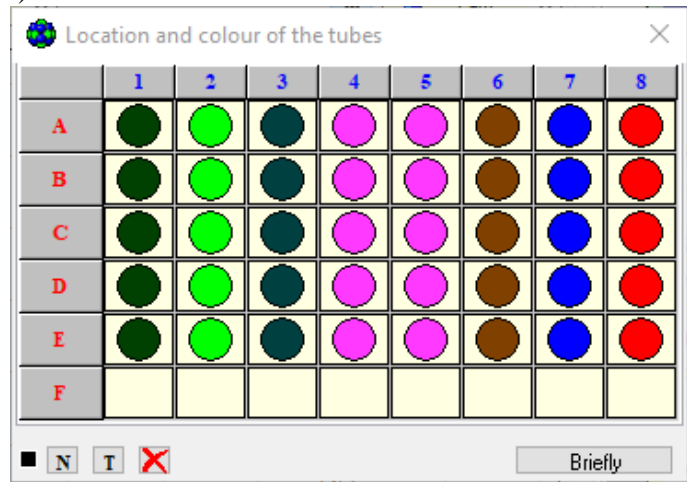8. 🔧 - export results as XML to LIS
9. ⚙ - show panel of device

Panel of device:
1. 📷 - opening and saving RUN measurements:
   - 📂 - open Run
   - 💾 - save RUN
   - 📄 - export results in xml format (old version)
   - 📄 - export results in txt format
   - 🔧 - Export of results in xml format to LIS
   - @ - sending RUN via email
2. 📊 - general information on the results of analysis and measurement data:

- location and color of the tubes (Fig.66).
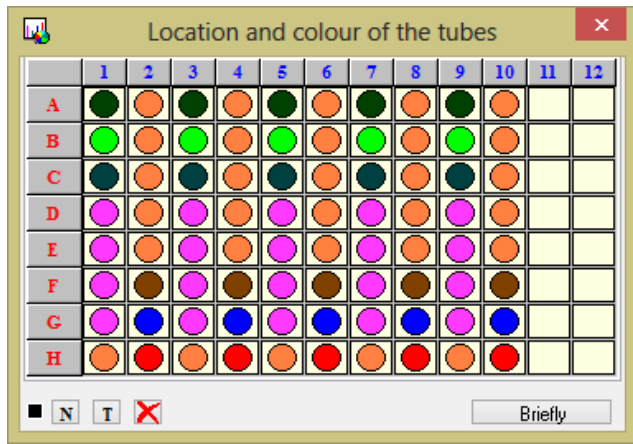
(For DTprime)   (For DTlite)

Fig.66 Arrangement and color palette of tubes

 - RUN information: amplification program, RUN file name, serial number of the device, etc (Fig.67).
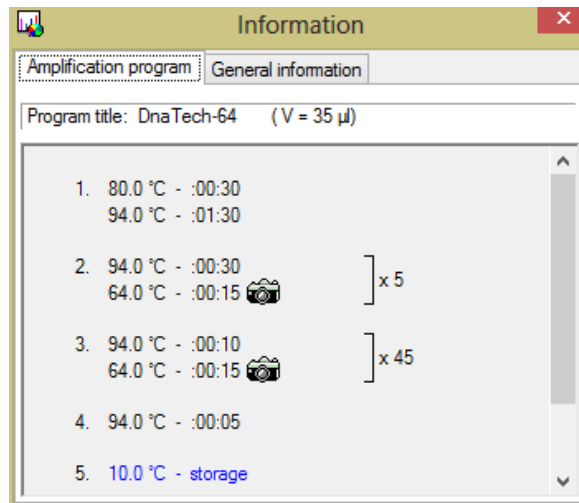

Fig.67 Information about the RUN

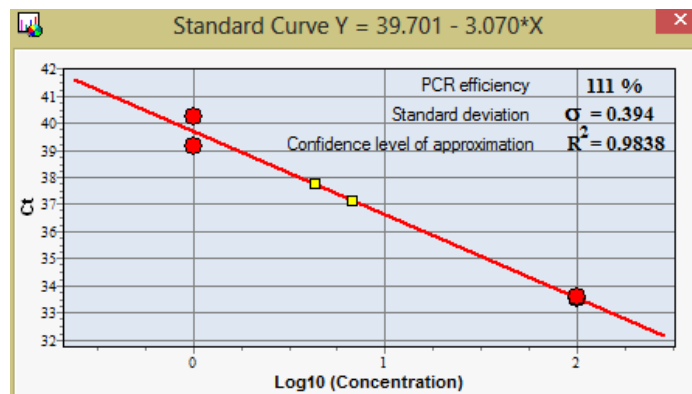 - standard curve for quantitative analysis (Fig.68).

Fig.68 View a standard curve for quantitative analysis
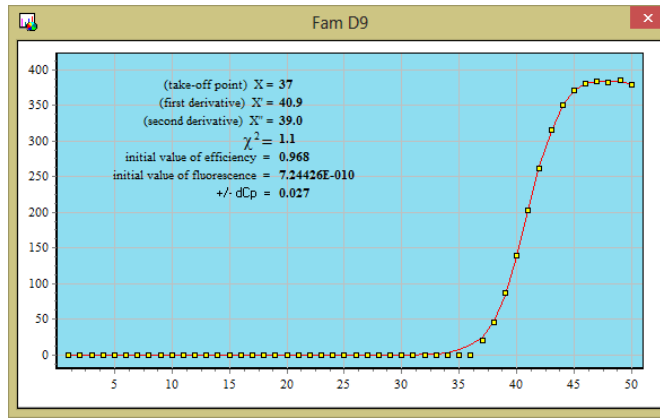
 - PCR-sigmoid fitting parameters (Fig.69).

Fig.69 The parameters of fitting of PCR- sigmoid

 - results of the analysis by the location in the thermal block (Fig.70).

(For DTprime)   (For DTlite)

Fig.70 The results of the analysis on the location in the thermal block

PCR - parameters of PCR curve analysis (Fig.71).
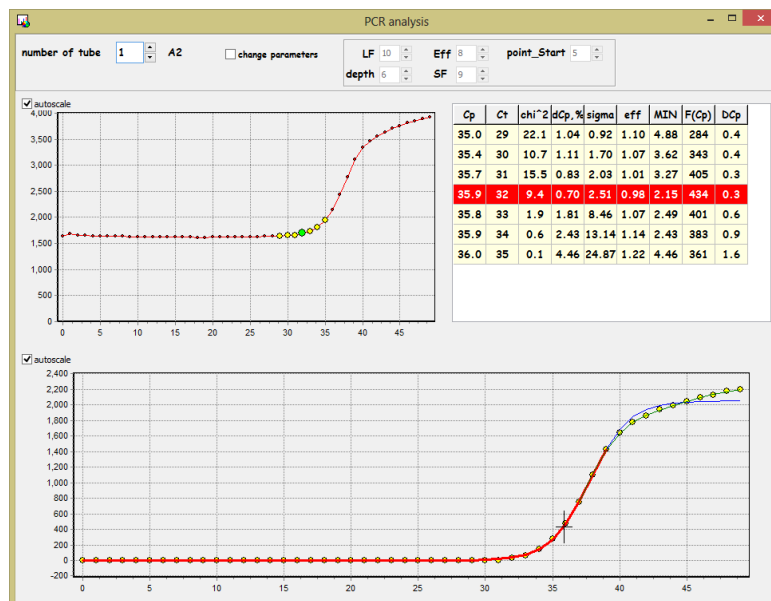

Fig.71 Parameters of PCR curve analysis
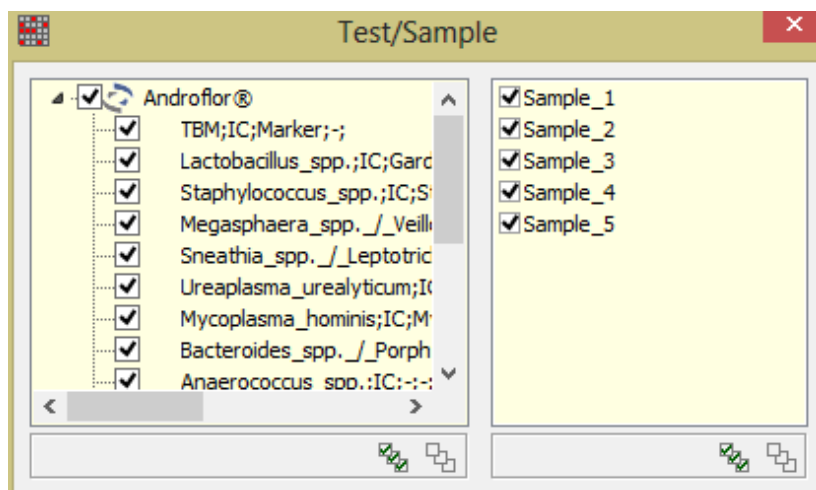
⊞ - display of tubes in the Test/Sample mode (Fig.72).

Fig.72 The display of test tubes in a Test/Sample

3. 𝗠𝗖 - analysis of the melting curve profile:
  🔲 - brings the coloring in accordance with the result of the analysis of the melting profile
  🔲 - shows heterozygote result
  🔲 - shows homozygote1result
  🔲 - shows homozygote2 result
  🔲 - shows a "doubtful" result
  ⊤ - shows the temperature marker
  🔲 - shows temperature limits
  🔲 - shows reference standards (for the corresponding analysis)
4. 🔲 - optical channel selection (option 2):
  🔵 - fam
  🟢 - hex
  🟡 - rox
  🟣 - cy5
  🔴 - cy5.5
  🔲 - all channels
5. 🔲 - Others:
  🔲 - calculating "negative" PCR
6. 📊 - shows the panel of studies analysis type: basic (academic) studies + studies that are present in the current RUN.

Additional panels required for work can be dragged to any place in the working window of the software using the mouse, and afterwards the toolbar can be removed.

The following elements can be used to change the way of results displaying in Archive window:
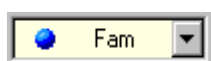
🔵 Fam ▼ – the window for selecting the displayed fluorescence channel (using the drop-down list).
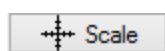
✛ Scale – the button displays an additional window **Scale of axes** for manual scale adjustment on the X and Y axes.

Auto✛ Off – automatic scale adjustment button.

Log_Y – the option to display the graph on a semi-log scale (it is recommended to turn off the fitting option ✖ that is located on the right corner above the graph).

Marker – buttons to change the size of markers.

Line – buttons to increase/decrease the thickness of lines.

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button to select the displayed tubes and their colors (**location and color of the tubes)**. Displays an additional window for performing this operation (Fig.73).

If necessary, select the color and number of lines in the fluorescence graph, press the button 🔳 (in the window **RealTime_PCR** tab **Running** or in the window **Archive** in the tab **Data analysis**). In the opened window **Location and color of the tubes**, the matrix of the thermal block displayed. The color of the tube corresponds to the color of the line in the graph. The number of tubes corresponds to the number of lines displayed in the graph.
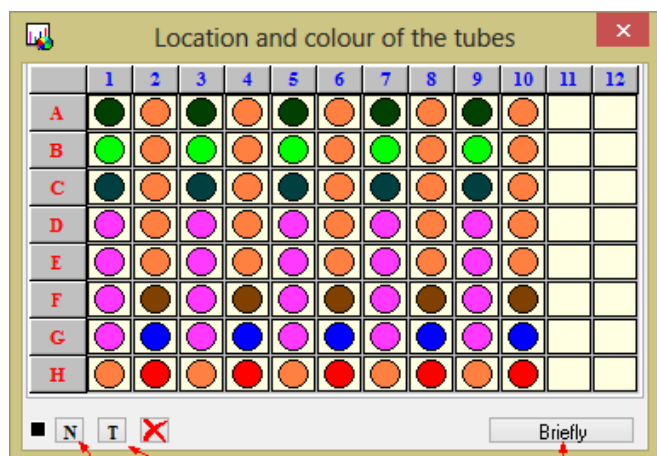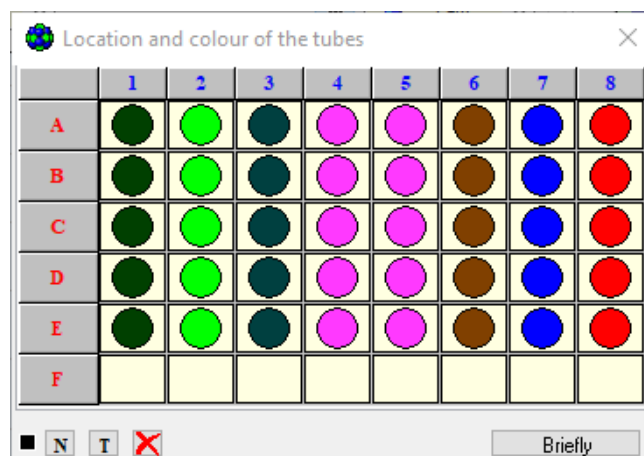
(For DTprime)   (For DTlite)

Fig.73 "**Location and color of the tubes**» window

To display the numbering of tubes according to the RUN, press the button **N**, to display the type of tubes press the button **T**.

To change the color of the tube, click right mouse button on the selected tube, in the opened **Color** window select the desired color and press **OK** (Fig.74).
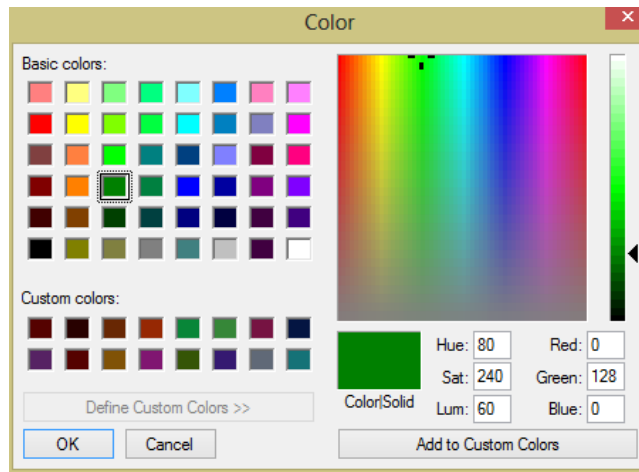
Fig.74 Choice of color

To select or deselect the tube, click left mouse button on the selected tube.

It is possible to select a group of column or row of tubes, for that procced the following:

- click the zero cell of the selected column or row , respectively,
- select the area (select the area holding down the left mouse button),
- "inverse" selection (show the hidden tubes and hide the displayed ones) - click on the upper left square of the table to show the hidden or hide the displayed tubes (Fig.75).

Fig.75 Show hidden tubes and hide displayed ones

In order to clear the entire field of the thermal block matrix, use the button ✖ (Fig.76).
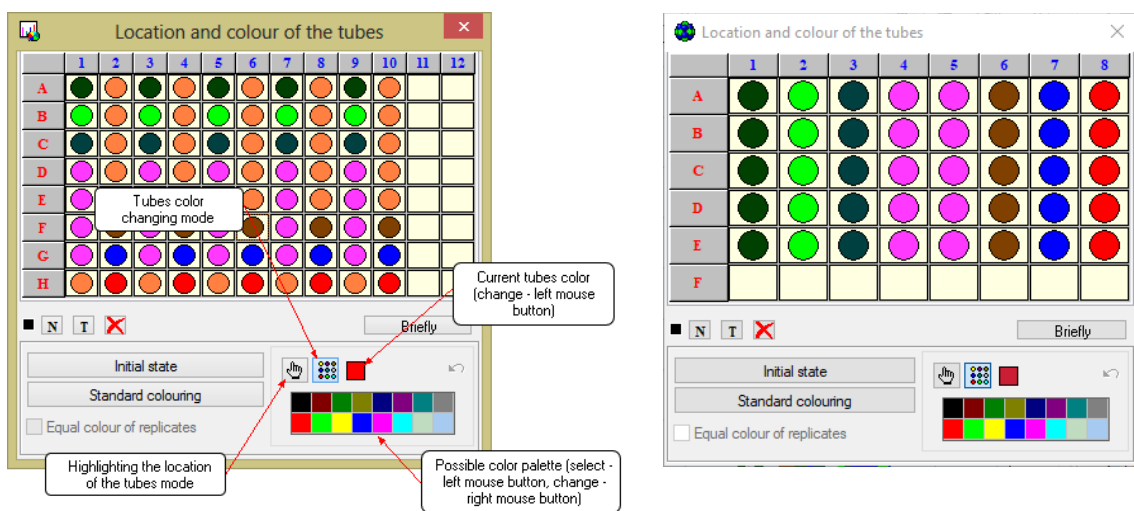
(For DTprime)   (For DTlite)

Fig.76 Expanded «**Location and color of the tubes**» window

To access additional color control buttons and the location of the tubes, press the button **Briefly** and then **in Detail**.

In the expanded window, additional buttons appear, with the help of which it is possible:

- to return to the initial state (initial location and color palette of the tubes);
- to designate an arbitrary color of tubes;
- to designate the same color for duplicates;
- to select the color of the tube from the preset color set;
- undo the last change in color.

If necessary, select the method for processing the measurement results

View the results of the settings either in All tests mode or by selecting one test from the drop-down list:

Tests: [All Tests ⌄]
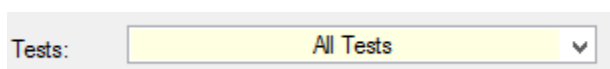
The following elements can be used to change the way results are processed:
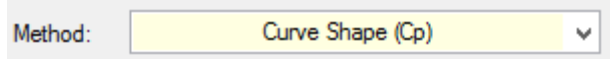
Method: [Curve Shape (Cp) ⌄] – selection of the analysis method.

By default, the analysis method specified when preparing to RUN using the **Test** procedure. If the RUN is prepared without using the **Test** procedure, the **Geometric (Cp)** method will be specified in the corresponded field.

– button is intended to open the window **Parameters of the analysis** (see **"Parameters of the analysis "**). The default values are preset and optimal. When conducting scientific research and for a more detailed analysis of the obtained data, change the preset parameters for processing the results of optical measurements and data analysis (Fig.77).
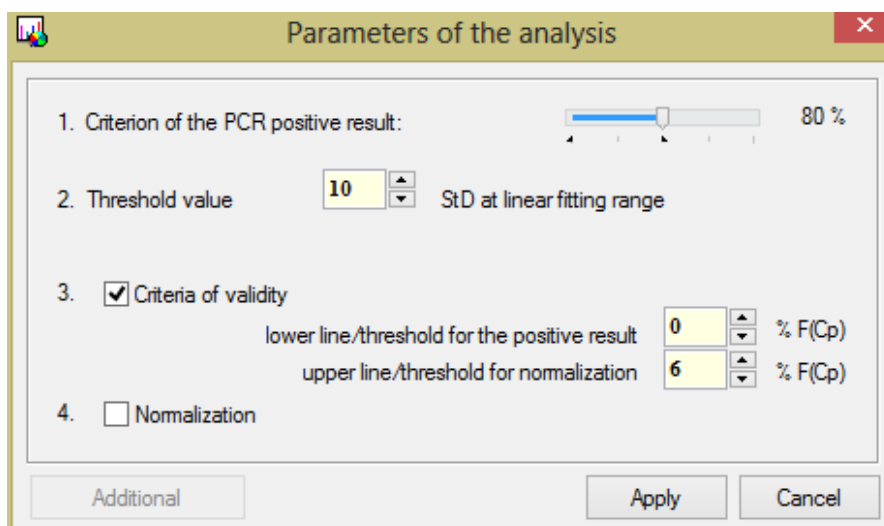
Fig.77 The analysis parameters

Parameters change is effective only in the framework of the current RUN.

– button is intended for switching on/off fitting (**smoothing**) of the PCR curves.

The **Fitting** option is applicable only after subtracting the "base" line for samples in which fragments of the desired DNA were found (for "positive" tubes). The button is not active when selecting the analysis method **Initial area** and when choosing the type of analysis **Melting curve**.

5.3.3 Creating report

By results of the measurements analysis, it is possible to generate a report.

The report includes the following data:

1. Analysis information:

    – date of the analysis;

    – protocol number;

    – operator;

    – name of the RUN file with the results;

    – comment to the protocol;

    – description of the amplification program;

    – the volume of the reaction mixture;

    – description of analysis settings.

2. Results of the analysis are displayed in the form of a table.

The following data can be added to the report:

1. Fluorescence graph for each fluorophore.
2. Statistical data for a series of duplicates.
3. Graph of the standard curve.
4. Location of tubes in the thermal block matrix.

To create a report based on the analysis results, perform the following actions:

1. Press the button ![icon] on the tab **Data analysis** in the window **Archive**. The **Report** window is opened (Fig. 78).

    With the help of the appropriate functions, the following options are available:

    ![icon] – place the page in the preview window entirely.

    ![icon] – place the page in the preview window by width.

    ![icon] – arrange two pages in the preview window.

    ![32 %] – change the scale of the preview window.

2. Select data to include in the report:

- – add fluorescence curves for the corresponding fluorescence channels;

- – include statistical data on series of duplicates in the report;

- – include a graph of the "standard curve" in the report (the option is active for analysis types **Quantitative with standards and GMI**);

- – display the location of the tubes in the thermal block matrix in the report;

- – include the data of additional standards in the report;

- – display the report in black and white;

- – include the display of results grouped by samples in the report.
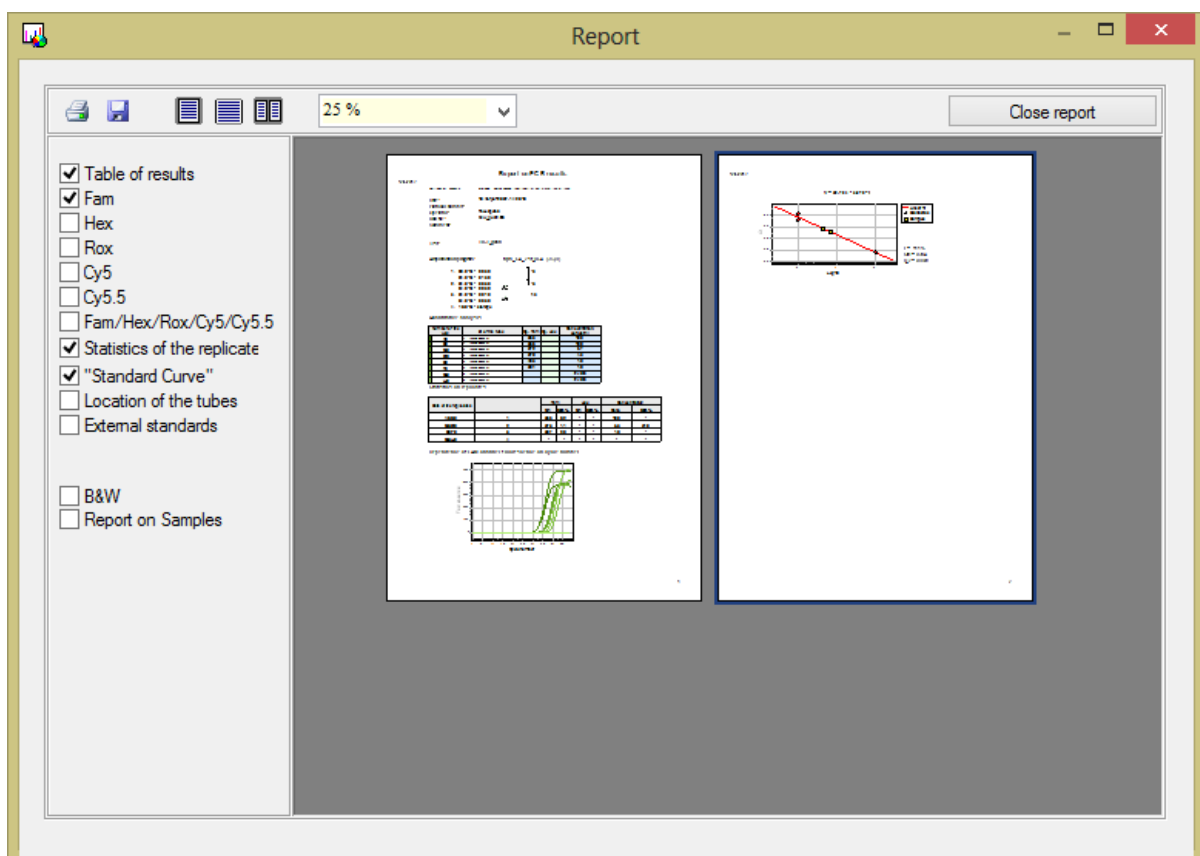
Fig.78 The "Report" window

3. Press the button to print the report.
4. Press the button to save the report to a file on the computer.

   - select the file format to save(Fig.79):
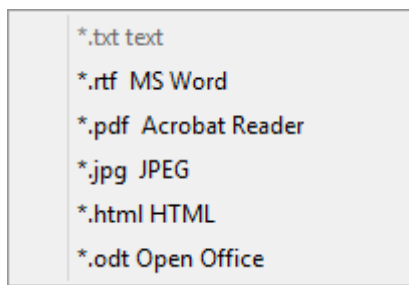
Fig.79 Format selection

– In the opened window **Save the analysis results as...** specify a file name and select a folder on the hard disk or any other device to which it will be saved (Fig.80). After clicking the button **Save** the file will be saved to the specified directory.
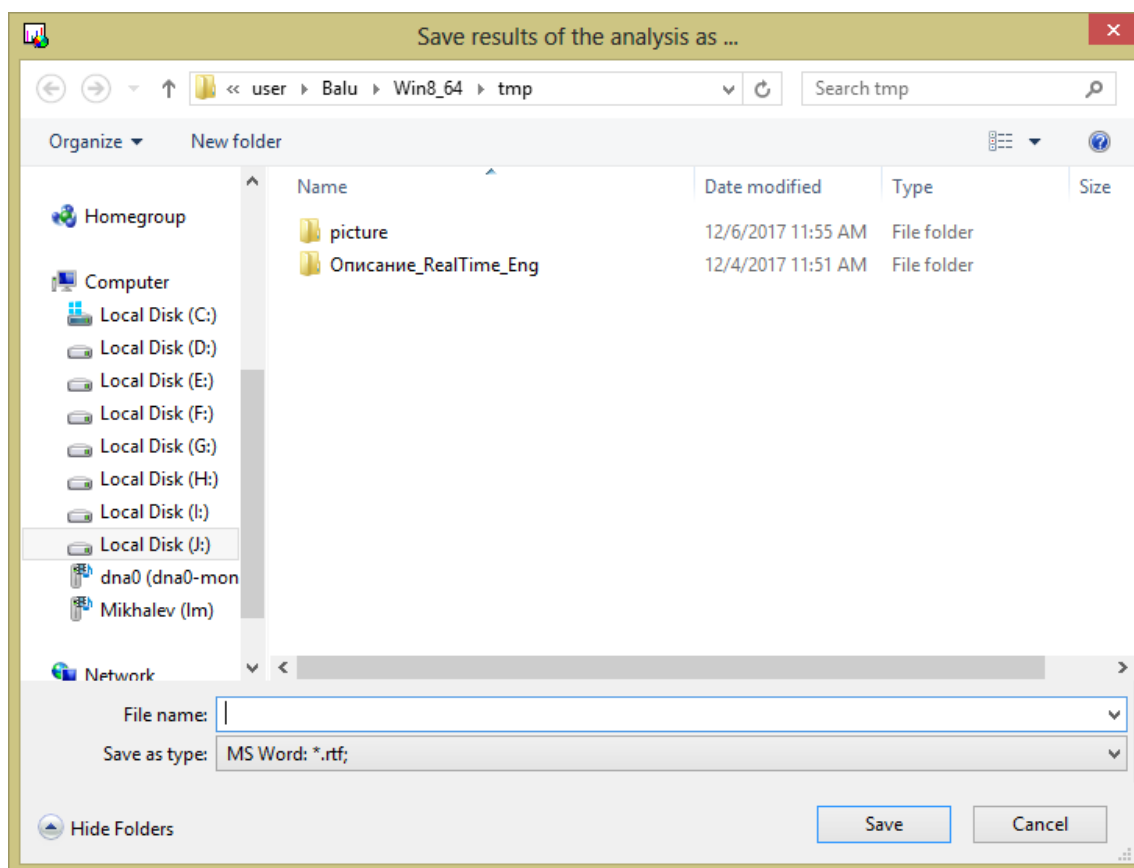
Fig.80 Saving the report

5.3.4 Export data

Export the data for transfer to the LIS using the button ⚏ **Exporting results as XML**. Report in XML format containing the calculated results in numerical form will be generated.

Also, the measurement results can be exported as an Excel table or copied to the clipboard. For this, in the "**Archive**" window choose the "Data analysis" tab and click right button of the mouse on the "Results" table (Fig.81).

Fig.81 Copy the contents of the table

Choose the way to export the data:

- – copy to the clipboard;

- – copy to Excel. Selecting this option, the "Explorer" window is opened, where it will be prompted to choose how and where to save the Excel data file.

6 Settings and diagnostics of the device.
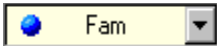
Settings of technical parameters of the device and its diagnostics are carried out in **Device operation mode**.

Using the RealTime_PCR software the following parameters can be checked and set:

- the geometric settings of the optical unit of the device;
- the purity of the wells;
- the height of the tubes;
- the optical measurements.

6.1 Checking the geometric settings of the optical unit and the purity of the wells.

When switching on the device for the first time after its transporation or any movement, it is recommended to check the geometric settings of the optical unit. For this, proceed the following steps:

1. Select item **View image** in the menu **Preferences** of the **RealTime_PCR** window.

2. In the opened window **View image** (Fig.82) using the drop-down list select the measurement channel **Fam**.

3. Set the required exposure value in the window **Exposure** to get enough of optical signal levels in the range of 1000 ... 3000 (the scale value when obtaining the digital values of the fluorescence signals in the window **Digitization of the signal**).

4. Press the button .

5. Make sure the checkbox is ticked.

6. Analyze the resulting image.
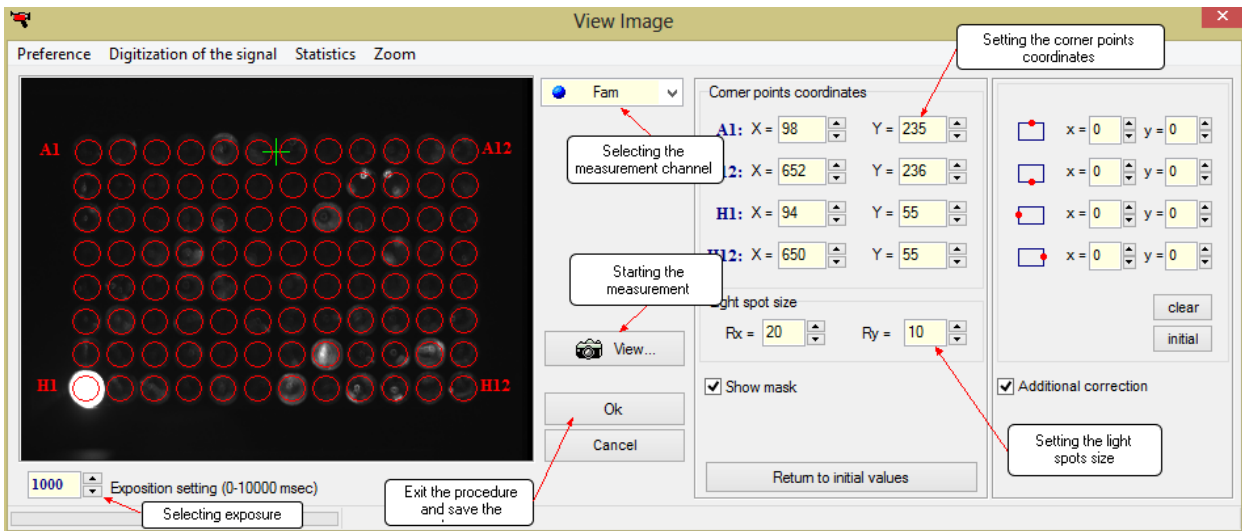
(For DTprime)
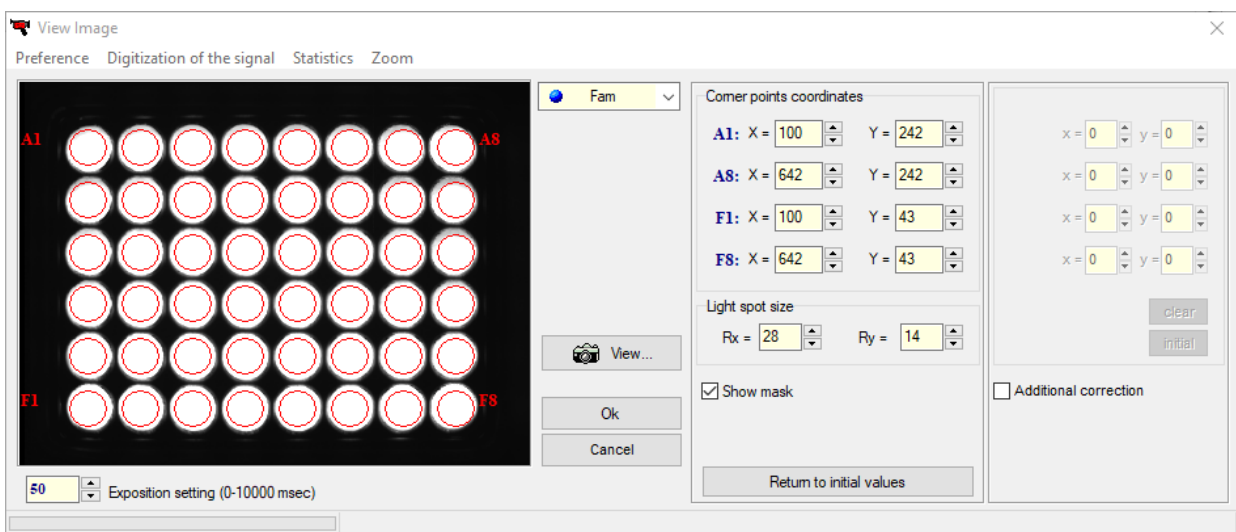
(For DTlite)

Fig.82 Window for checking and correcting geometric settings of the device

In the resulting image, the contours denoting the boundaries of the measured area (red circles) should not go beyond the light spot.

If the center of the pixel constituting the light spot is inside the red circle, it is taken into account during the measurement. Pixels whose centers are outside the boundary of the red circle will not be taken into account (Fig.83).
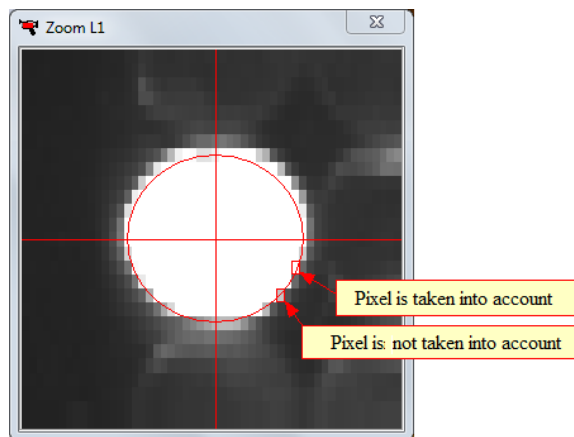
Fig. 83 Measurement area for fluorescence signals

If the circles are offset relative to the light spots, it is necessary to correct the geometry of the optical image.

7. To correct the geometry of the optical image in the Image preview window, select the point to be corrected with the left mouse button. For more accurate correction, press the button **Zoom**. A window will appear that zooms the image of the selected point.

8. Correct the coordinates of the corner points and the size of the light spot by changing the values in the corresponding windows.

   Note! To cancel the changes that are made press the button **Return to initial values**.

9. Press the button [📷 View...] for re-measurement.

10. Press the button **OK** to save new geometric settings.

11. Successively select the remaining channels of the device, and by setting for them in the same way the value of exposures, repeat these measurements for each of them. Control the position of the measuring mask and scatter of optical signal levels.

   Caution! Adjustment of measuring mask position is performed only in **Fam** channel, during it the position of the measurement mask automatically adjusts in the other channels.

12. If the curvature of the image for the 384-well device is expressed, continue the correction and set the offset of the central coordinates (Fig.84). The central coordinates are the centers of the sides of the perimeter forming. During offset correction, the central point, which coordinates are corrected at that time, is indicated on the image with a green cross [image].

   In order to enter the offset coordinates of the corresponded fields tick [✔ Additional correction].
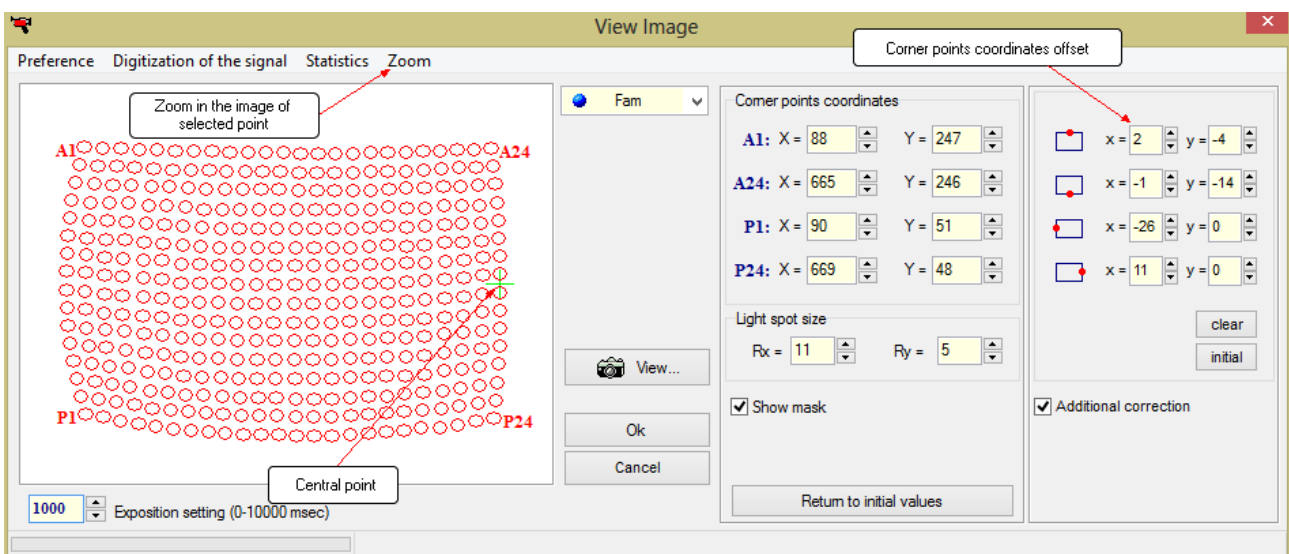
(For DTprime)
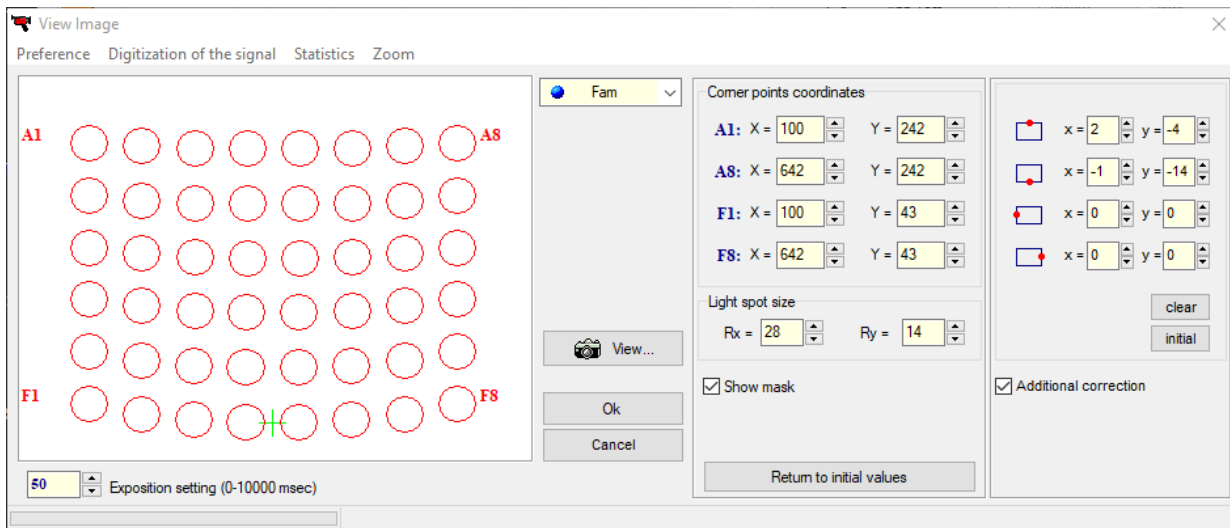
(For DTlite)

Fig.84 The window for adjusting the geometric settings of the device, adjusting the displacement of the coordinates of the central points.

To reset the offset coordinates to zero position, press the button clear , to return to the previous values press the button initial .

13. To obtain the digital values of the fluorescence signals, select the option **Digitization of the signal** for displaying an additional window **Digitization of the signal** (Fig. 85).
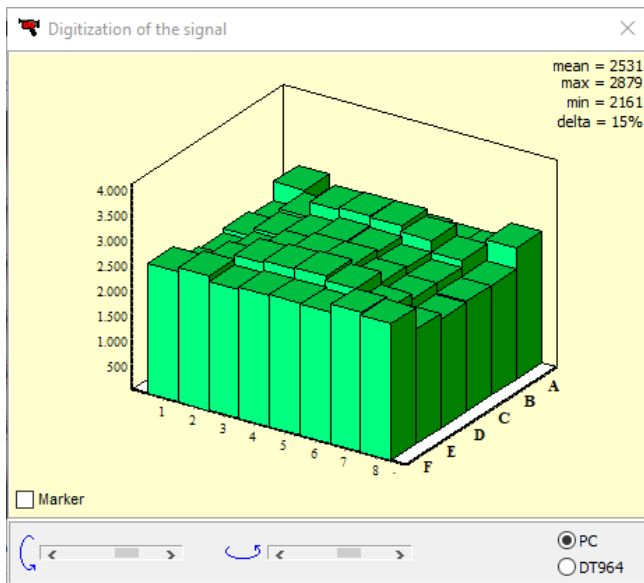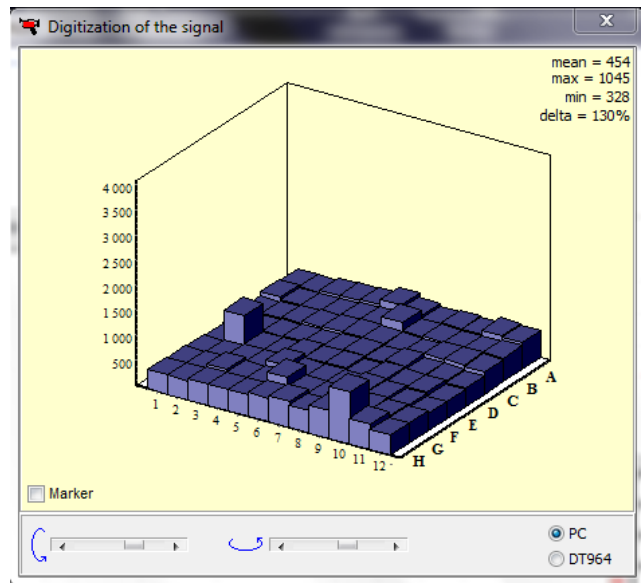
(For DTlite)   (For DTprime)

Fig.85 **Digitization of the signal** window

The option **Marker** allows displaying numerical values of measurements for each tube.

Selection of the option **Statistics** opens the window **Statistics of optical measurement** (Fig.86) with an indication of the time duration of the optical measurements and the average value of the video signal of the selected channel.
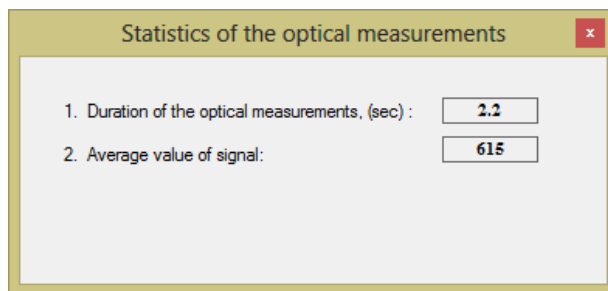
Fig.86 **Statistics of optical measurement** window

14. Repeat step 5-8 for measuring channels **Hex, Rox** and **Cy5** to make sure the settings are correct.

The **cleanliness of the thermal block wells** is indicated by the absence of the bright spots on the image of the empty wells when checking the geometric settings of the optical unit (fig 87).
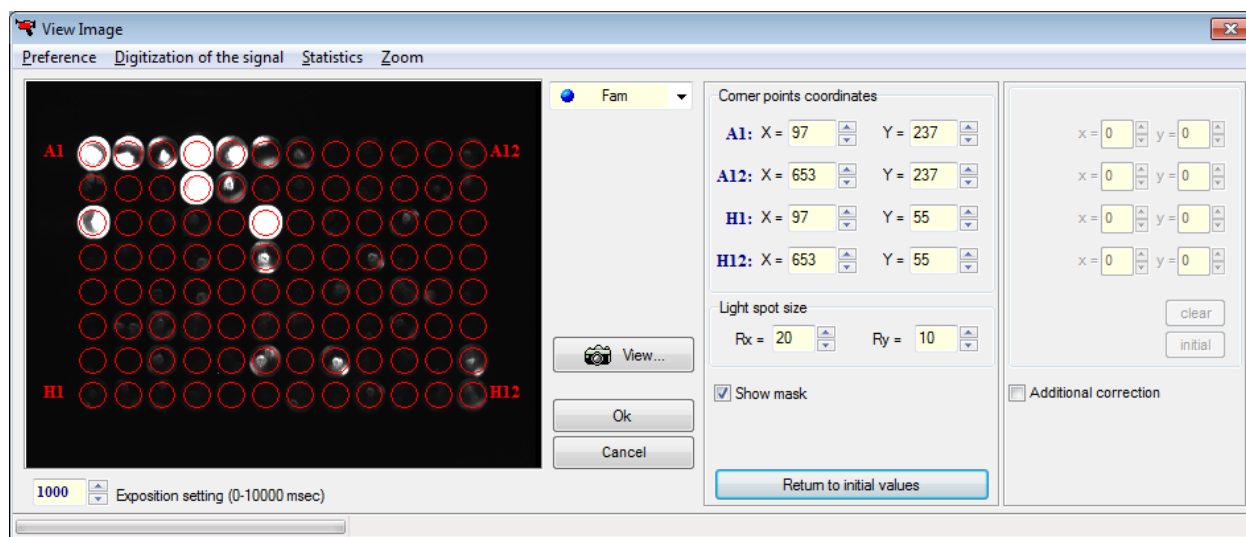
Fig. 87 Example of checking the cleanliness of the thermal block wells

To check the cleanliness of wells, and the lack of stray reflected optical signals perform the following:

1. Close the thermal block, make sure there are no tubes in the wells.

2. Open the window View Image (Preference/View image), choose Digitalization of signal and open this window, set the exposition.

3. Press the button [📷 View...], start the process of optical measurement in FAM channel (selected by default).

4. Make sure that the optical signal levels for empty wells in the Digitization of video signal window (see fig.88) does not exceed 1500 conventional units, otherwise clean luminous wells and repeat the measurement.
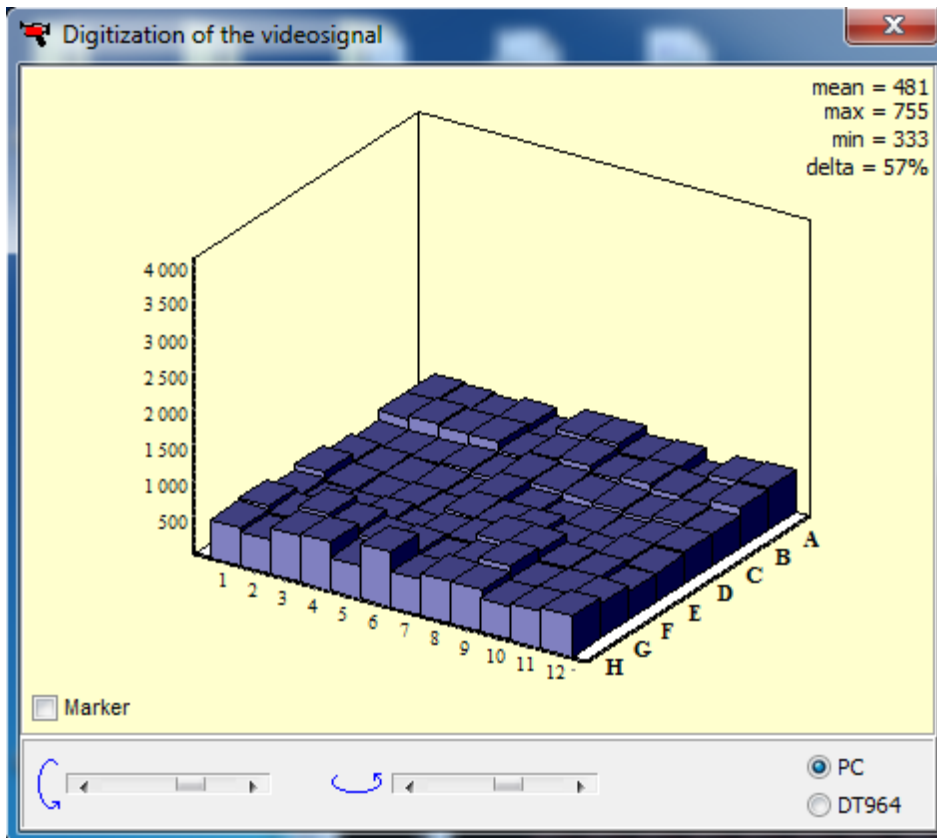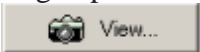
a)
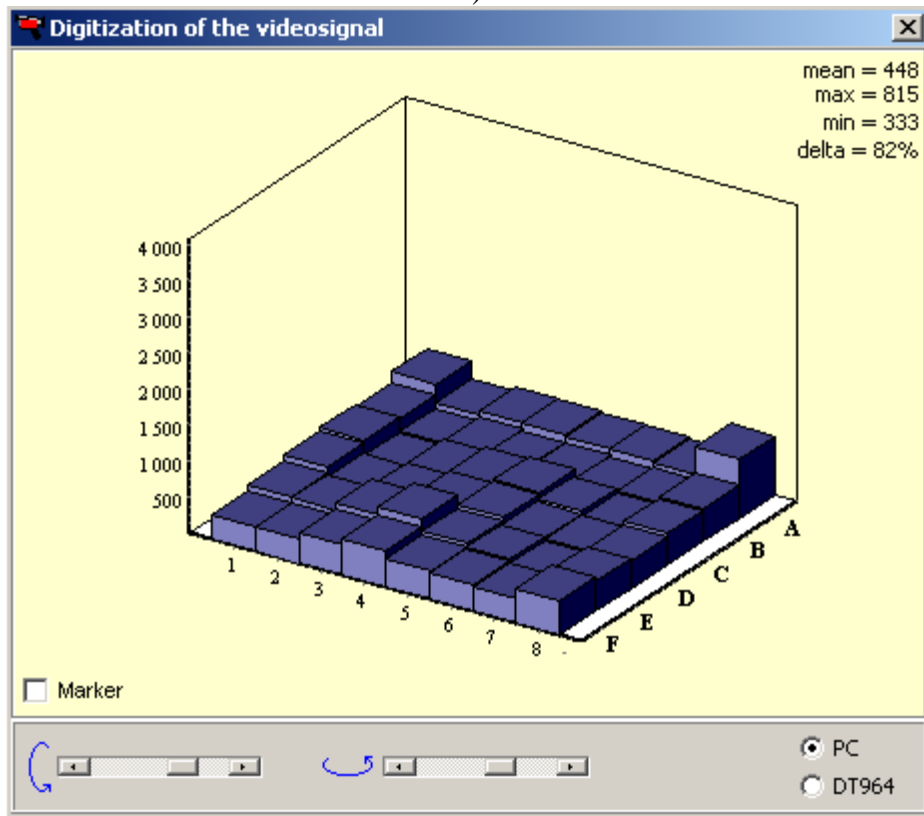
b)

Fig.88 The view of empty wells DTprime (a) and DTlite (b) devices

5. Successively choose the rest channels of the device and set for the exposition values (twice bigger than the working exposition values), repeat these measurements for all channels (by

pressing the button [📷 View...] and controlling the levels of optical signals in the Digitization of video signal window).
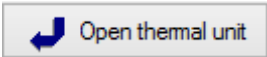
Note! It is recommended to check the purity of the wells immediately before placing tubes with PCR samples if there is any possibility of contamination.

The regular check of the purity of wells should be carried out by the operator in accordance with the device maintenance recommendations.

6.2 Measuring the height of the tube

Measurement of the height of the tube is obligated when changing to a different type of plastic consumables (tube, strips, plates) or when there is a doubt about the correct pressure on the tubes by the "hot lid".

To measure the height of the tube, proceed as follows:

- click the button [Open thermal unit] and place the tubes or strips evenly over the thermal block matrix;
- select the Preferences/Device diagnostics/Measure height of the tubes (Fig 89), and wait for the message (Fig.90):
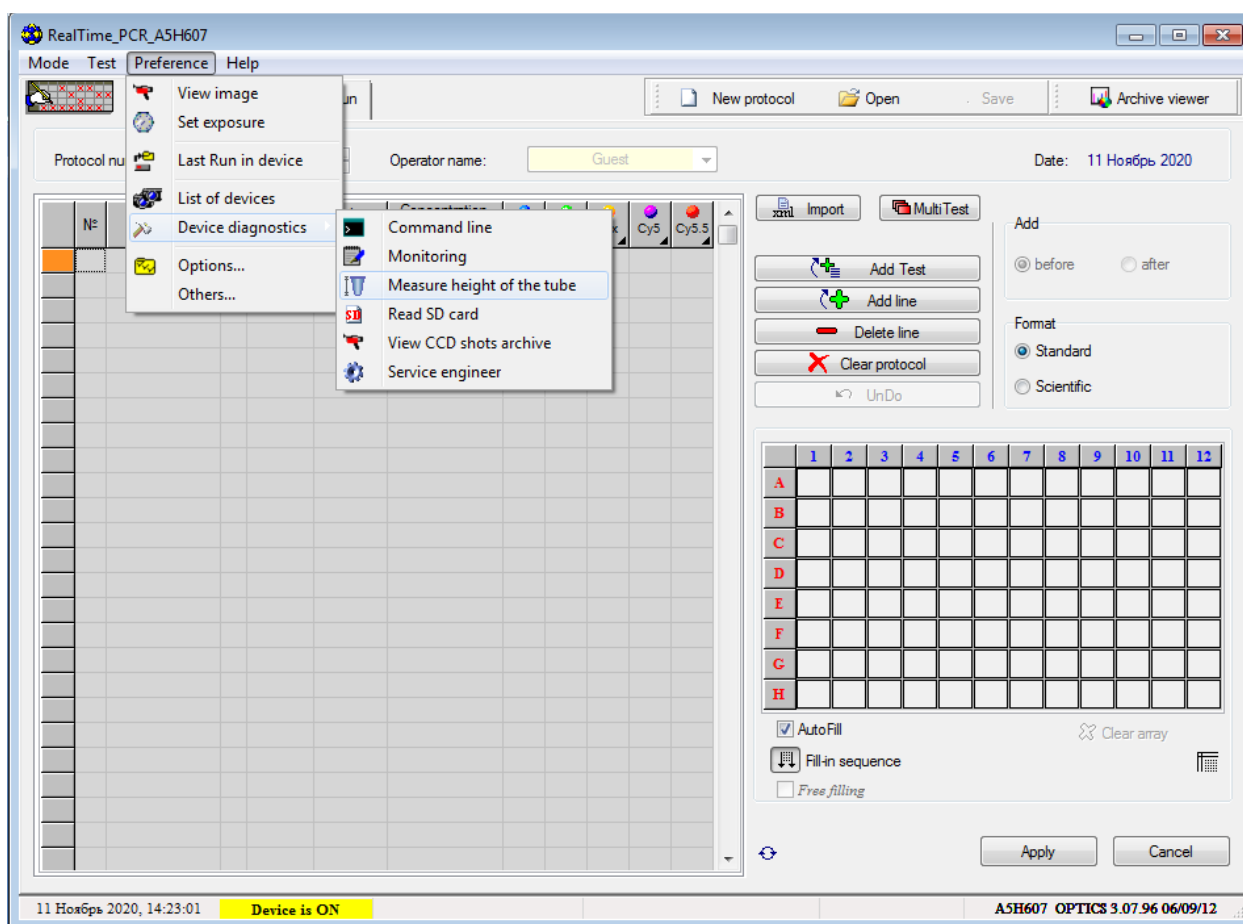
Fig.89 Measure height of the tubes
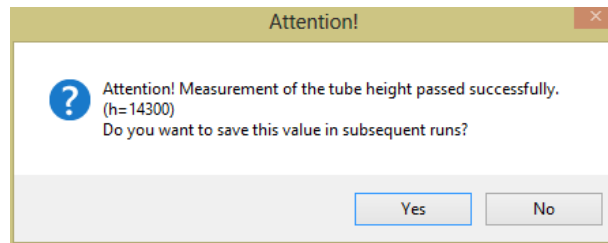
Fig.90 Successful change of test tube height

-   save the measured height of the tube by pressing the button [Yes]

Attention! When measuring the height of the tube, place at least 32 test tubes or 4 strips evenly over the thermal block matrix.

6.3   Setting the exposure of optical measurements

To set the exposure of optical measurements, select item **Set exposure** in menu **Preferences** of the window **RealTime_PCR**. A window **Exposure** will appear (Fig. 91).
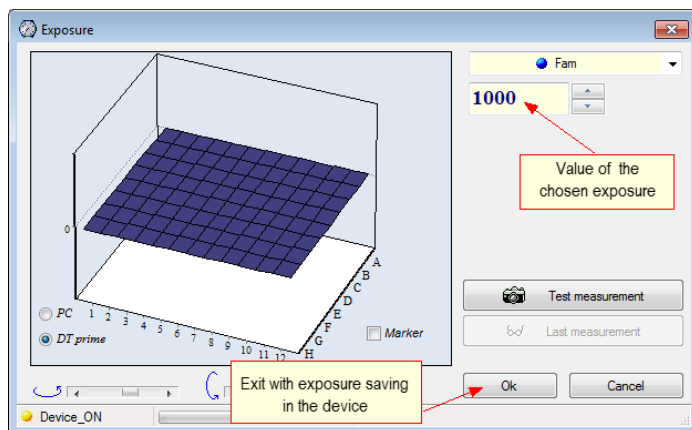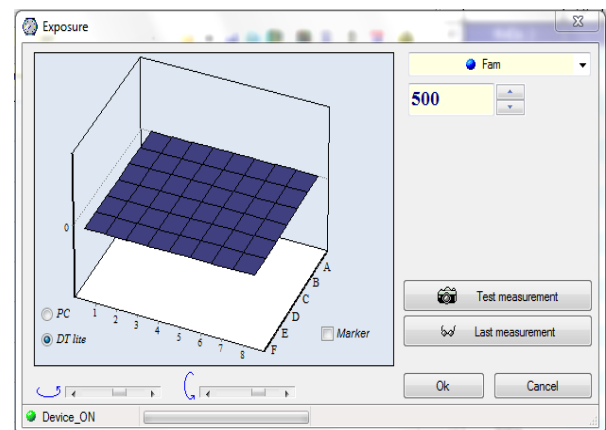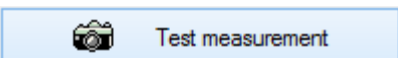
(For DTprime)   (For DTlite)

Fig.91 The window for setting the exposure of optical measurements

Preset exposure parameters of optical measurements are optimal for DNA-Technology PCR kits. The change of exposure parameters could be required if using PCR kits from other producers.

If necessary, set the exposure value for each channel. It is obligatory to set the exposure of optical measurements for all channels by selecting them sequentially in the upper right corner of the window **Exposure**.

If there is a fluorescence source, perform a test measurement by pressing the button [📷 Test measurement], as a result of which a graphic image of the current values of optical measurements in three-dimensional coordinates will be displayed in the left part of the window.

The buttons with arrows under the diagram allow rotating the image in the horizontal and vertical planes, the option **Marker** allows displaying numerical values for each tube.

Note! The red color of the column warns that the fluorescence value in this thermal block well is above the linear measuring range.

In order to expand the optical range, each measurement can contain from 1 to 2 exposures for DTprime and DTlite (the latter is five times shorter than the first). In this case, the maximum exposure time is set in the window **Exposure**. The number of exposures can be adjusted in the menu **Preferences/Options/Optical data acquisition** (Fig. 92).
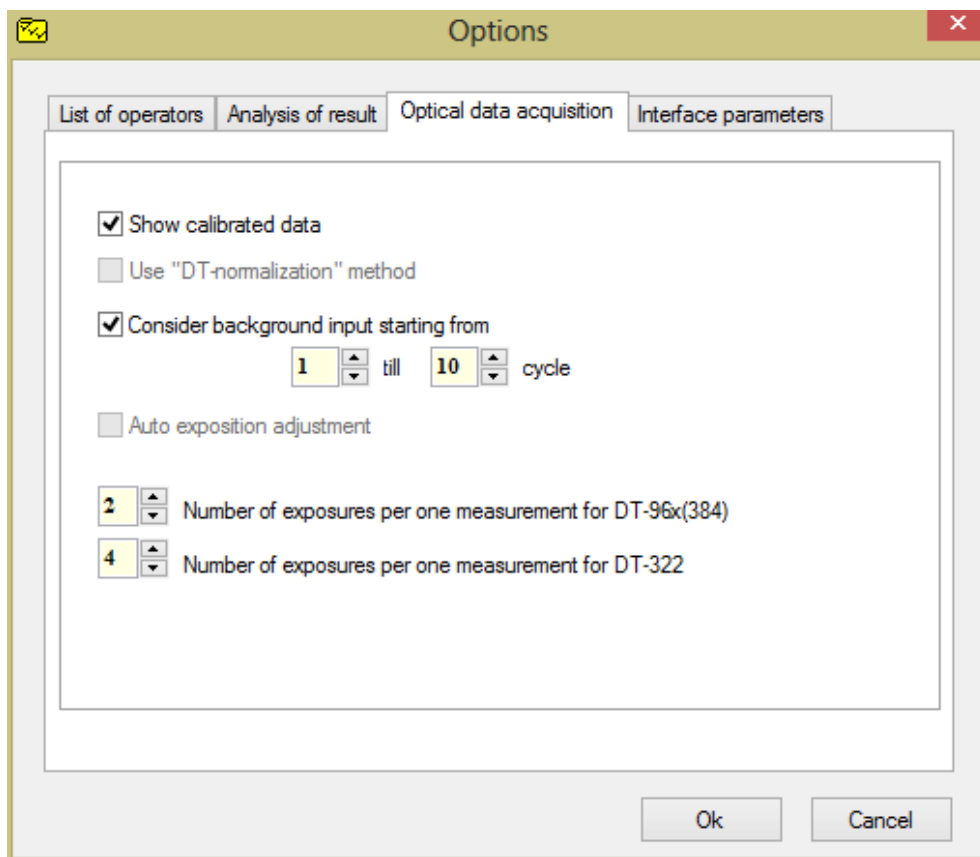
Fig.92 Setting the number of exposures

6.4 Color compensation in DTPrime and DTLite

The cross-channel effect, arising from a partial overlapping of the absorption and emission spectra of fluorescent dyes, is eliminated by the software method. For this purpose, the matrices of the optical and spectral coefficients obtained by calibrating of the device with pure fluorescent dyes are stored in the device memory.

Attention! The color compensation works only if the plate protocol is filled using the procedure ⟨Add Test⟩, that is, each tube corresponds to a specific test, which indicates the composition of the dyes included in the PCR kit used. Otherwise, the compensation will not be performed correctly.

6.4.1 Enabling signal compensation

Color compensation is enabled by default, as can be verified by selecting **Others…** from the menu **Preferences** (Fig. 93).
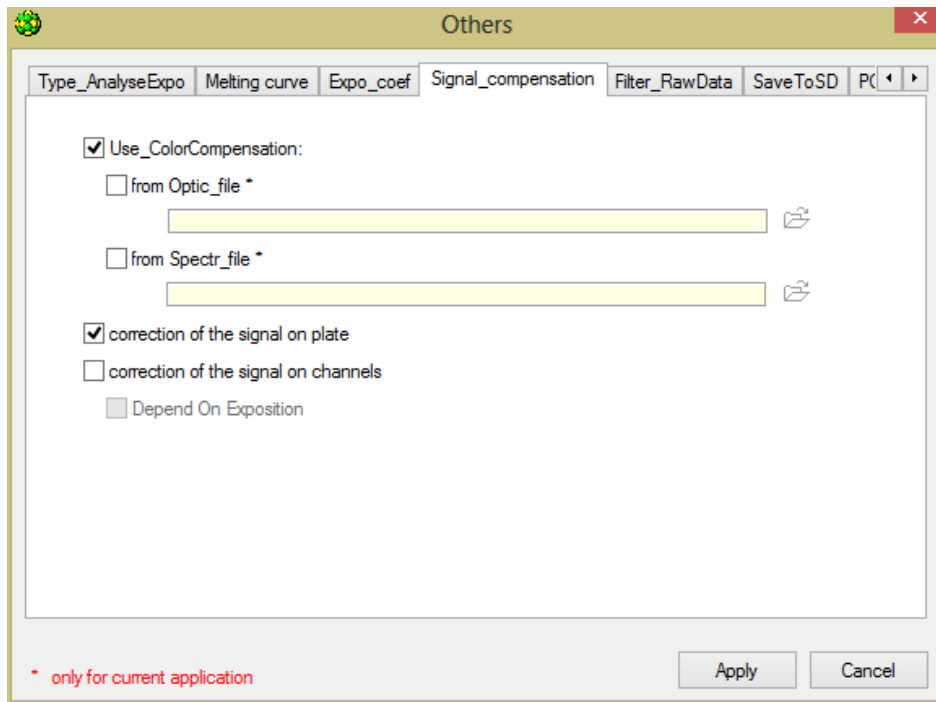
Fig.93 Enable signal compensation in the Others window

In the opened window **Others** on the tab **Signal compensation** the **Use_ColorCompensation** must be enabled. If this option is disabled, the color compensation will not be applied.

6.5   Diagnostics of the device

The device is diagnosed by a service engineer during the installation and maintenance procedure.

Attention! **It is not recommended** to use this diagnostic service options by the end user.

To diagnose the operation of the device, select **Device diagnostics** from menu **Preferences** of the **RealTime_PCR** software window:

–   to enter the diagnostic commands for the device, select item **Command line**. The **Command string** window will be opened (Fig.94). Buttons V T M D are intended for the selection of the controller, the diagnostics of which has to be performed;

Fig.94 Command string

– to display the diagnostic information, select item **Monitoring**. The **Monitoring** window will be opened (Fig.95).
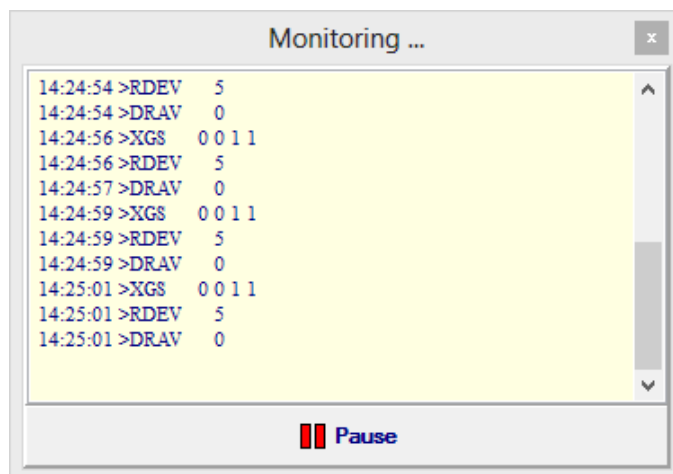
Fig.95 Monitoring

7   User settings

In a **RealTime_PCR** software it is possible to save individual settings for each user (operator) of the software: operator name, a folder for saving files, font style, window presentation style (only for Windows XP operating system).

To change the user settings, open the window **Options...**   (select item **Options...** in the menu **Preferences**).

Tab **List of operators** of the **Options** window (Fig.96) allows performing the following actions: adding, editing and deleting operators, assigning a folder to save files.

– to add a new operator, use the button **Add**;

– to edit the operator selected in the list, use the button **Change**;

– to remove the selected operator from the list, use the button **Delete**.

After clicking the buttons **Add** or **Change**, the **Add operator window** will be opened, in which specify/edit **Operator name** and **Directory** (folder) for saving files (for more details, see "Starting the RUN ").
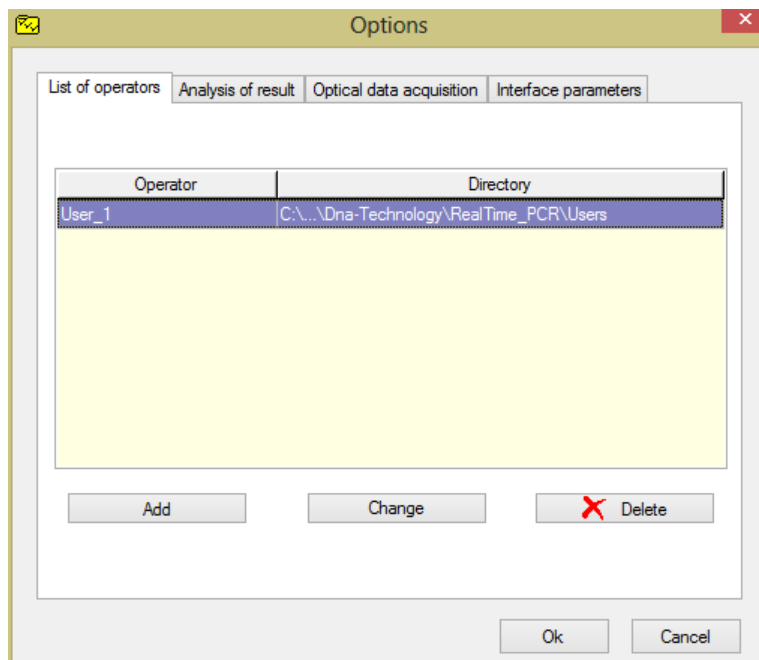
Fig.96 List of operators tab of the Options window

Tab **Interface Parameters** (Fig.97) is intended for selecting the font of the software and the language.
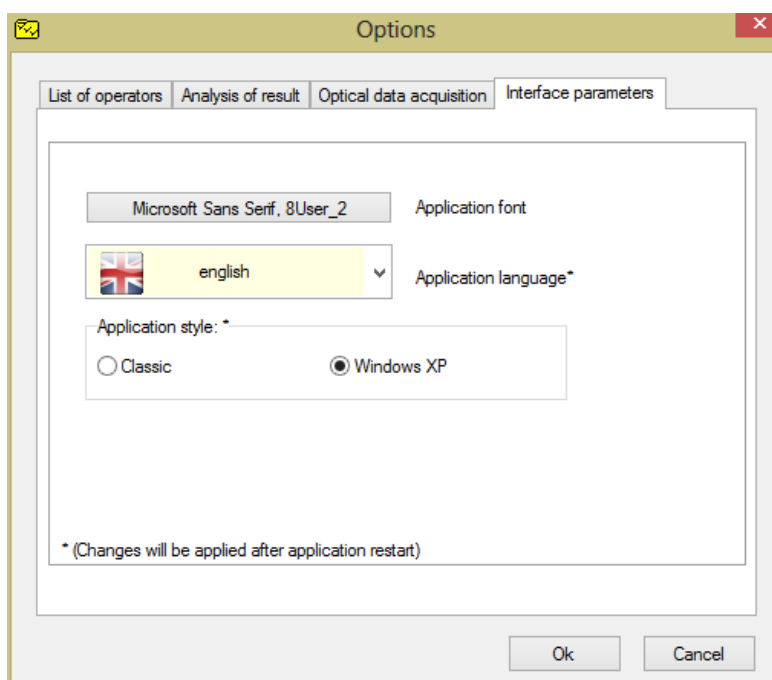
Fig.97 **Interface Parameters** tab of the Options window

8   Parameters of the analysis settings

Analysis parameters, **set by default**, are optimal for daily operations. When conducting scientific research and for more detailed analysis of the obtained data, change the preset parameters for processing the results of optical measurements and data analysis.

Attention! When working with commercial kits, the settings of analysis and device settings have to be requested from the kit manufacturer.

Parameters change is available at various stages of the software:

1. When adding/editing tests (button **Additional** of the window **Test**).
2. When creating a plate protocol (item **Options** of the menu **Preferences,** tab **Analysis of results**).
3. When starting and executing the amplification program (item **Options** of the menu **Preferences**, tabs **Analysis of results** and **Optical measurements**).
4. When analyzing the measurement results (tab **Data analysis**, button **Change parameters of data analysis**) - only for the analyzed RUN.

In this case, a window **Parameters of the analysis** is displayed (Fig.97) and the following settings are available:

- **Criterion of the PCR positive result** (in the range from 60 to 100%) allows changing the sensitivity to the rate of growth of PCR products. By decreasing the value of this parameter, visualization and calculation of more gently sloping curves can be achieved;
- **Threshold value** allows establishing a "threshold" level of fluorescence when determining the indicator cycle (it is considered in the threshold method of curve analysis);
- **Criteria of validity** of results are the parameters that allow adjusting the conditions for calculating the curve under the geometric method of analysis:
  - the threshold of the positive result is the lower limit;
  - the threshold of data normalization is the upper limit.
- **Normalization:** the use of the "amplitude normalization" method.
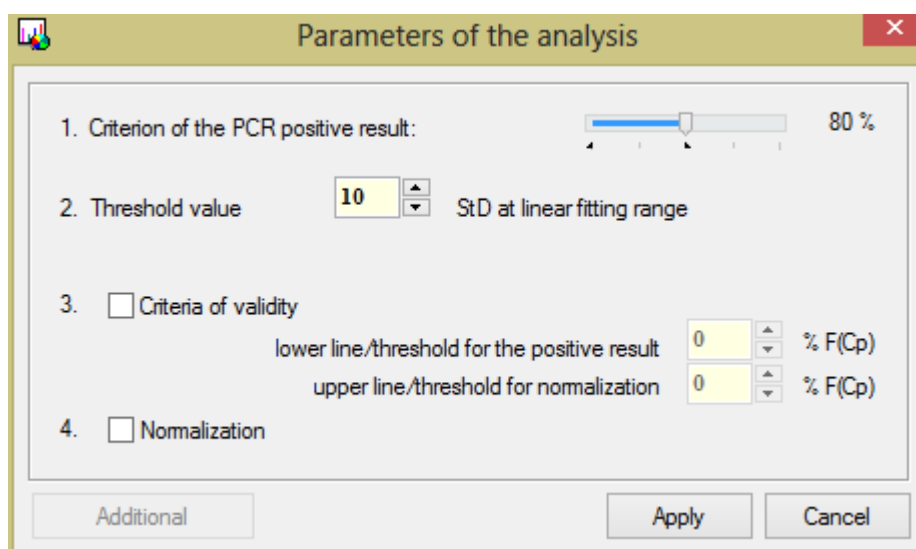
Fig.97 Parameters of analysis window

Method of analysis is selected during the Test creation stage or directly in the Analysis of optical measurements window.

Threshold (Ct) and Geometric (Cp) methods for analyzing the DNA accumulation curve during PCR are based on different approaches to determining the indicator cycle of amplification.

- **The Threshold (Ct) method** is based on conducting a threshold line that is parallel to X line and determining the cycle number (threshold cycle), at which the amplification curve for this tube crosses the threshold line. Threshold cycle is one of the variants of indicator cycles;
- **Geometric method (Cp)** is based on a mathematical analysis of the shape of the DNA accumulation curve during PCR.

By default, when analyzing optical measurements, RealTime_PCR uses the Geometric method (Cp). In case of non-standard situations, when the amplification curve differs significantly from the classical DNA accumulation curve in the course of PCR (sigmoid), it is possible to analyze the results using the Threshold method (Ct), which, in such cases, gives a better estimate.

Along with the processed results (deduction of the baseline, fitting), when analyzing the results of optical measurements, it is possible to view *raw data*, selecting the appropriate position in the drop-down list of analysis methods.

9   Emergency situations

❌	Attention! There was a malfunction when the device was initialized! Try to repeat the selected operation...	The device identification failed when connecting to the PC. It is necessary to repeat the procedure of connecting to the device...
❌	Attention! An error occurred while reading the optical mask of the device... You need to restart the application!	An error occurred while reading the setting parameters from the device. It is necessary to repeat the procedure of connecting to the device...
❌	Attention! An error occurred while reading the parameter block from the device... You need to restart the application!	
❌	Attention! An incorrect exposure value when reading the parameter block from the device is detected... It is necessary to check the exposure values and correct if necessary!	The validation of the parameter block from the device revealed the presence of incorrect values of the individual parameters of the tuning factors: exposure, coordinates of the optical geometry, etc. It is necessary to correct these parameters and save them again in the device. Contact the technical support of the manufacturer.
❌	Attention! An incorrect coordinate value of the geometrical setting of the optical image was detected when reading the parameter block from the device... It is necessary to check the geometric setting values and correct if necessary!	
❌	Attention! An incorrect value of the geometric adjustment coordinate offset is detected... It is necessary to contact the service engineer of the manufacturer!	
❌	Attention! An error occurred while reading the protocol from the device... It is necessary to repeat the reading procedure!	When starting a new protocol or connecting to device in which the protocol is already running, the protocol reading/writing failed, respectively. It is necessary to repeat the read/write procedure.
❌	Attention! An error occurred while writing the protocol to the device... It is necessary to repeat the writing procedure!	
⚠	Attention! An incorrect value of the coefficients of optical nonuniformity is detected... It is necessary to contact the service engineer of the manufacturer!	The validation of the optical coefficients of the device revealed the presence of incorrect values. Please contact the technical support of the manufacturer.
⚠	Attention! An incorrect value of the spectral nonuniformity coefficients is detected... It is necessary to contact the service engineer of the manufacturer!	
⚠	Attention! An incorrect value of Unequal Nonuniformity Coefficients has been detected... It is necessary to contact the service engineer of the manufacturer!	
❌	Attention! An error occurred while writing the protocol data to the device!	A glitch occurred when writing data to the idevice at the SD card level. Please repeat the writing to the device procedure.
❌	Attention! The temperature gradient/difference does not correspond to the temperature block of the device! It is necessary to make appropriate changes in the amplification program...	The temperature gradient/difference in the amplification program loaded into the device does not correspond to the temperature unit of the device, or MAY NOT correspond (in the case of old device, the information about the thermal block is missing). It is necessary to make appropriate changes in the amplification program...
⚠	Attention! The temperature gradient/difference may not correspond to the temperature block of the device! Do you want to continue the program?	

⚠	Attention! Incorrect values of geometric settings are detected... Continue the saving procedure?	The validation of geometric settings in the "Preview Image" procedure revealed the presence of incorrect values.
⚠	Attention! Problems when choosing colour-filters (MBUSY)...	During the acquisition of the optical picture in the "Preview Image" procedure, there was a malfunction in the device when the color-filter was selected. Repeat the procedure for reading the optical image.
⚠	Attention! Incorrect values of exposure values are detected... Continue the saving procedure?	The validation of exposure values in the procedure "Exposure of measurements" revealed the presence of incorrect values.
⚠	Attention! During the execution of the program, errors were found while storing the measurement values in the results file. It is necessary to consider the last run in the device (the corresponding item in the menu) immediately after the program is executed in the device!	During the execution of the protocol, errors in the procedure for recording optical measurement data in a file with results were detected. It is necessary to read the last start in the device immediately after the program is executed in the device.
⚠	Attention! There were troubles reading the part of optical measurements data from the device! Upon completion of the amplification program, it is necessary to read the last start from the device...	
❌	Attention! An emergency stop of the program has occurred!	During the execution of the amplification program in the device, an emergency stop of the program occurred. Please contact the technical support of the manufacturer and provide log files (if possible).
❌	Attention! An emergency has been detected in the device!	At the end of the amplification program in the device, an emergency situation was detected. Please contact the technical support of the manufacturer and provide log files (if possible).
⚠	Can not download the protocol! A discrepancy between the test list and the test in the protocol was found!	When creating a protocol and using an already existing protocol as a template, the list of tests for the operator and the list of tests in the template protocol is validated.
⚠	Attention! An inconsistency between the current list of tests and the list of tests in the last run was detected! Check the name of the operator under which the last run was read...	When reading the last run, the device found a mismatch in the tests in the read protocol and with the list of tests of the current operator, under whom the procedure for reading the last run is started.
⚠	Attention! The block is not in working order!	It is necessary to perform the procedure of opening/closing the thermal block.

Attention! In case of contacting the customer support service, provide the following:

1. The exact content of the error message.
2. The RUN file during which an emergency situation is occurred.
3. Log files that are located at C:\Program Files (x86)\Dna-Technology\RealTime_PCR\LOG.

Appendix 1. Glossary of Basic Terms

Thermocycler, Instrument, Device – the device, intended for polymerase chain reaction (PCR); it is a programmable thermostat capable of heating and cooling a tube with a reaction mixture according to a certain program.

Amplification of DNA – multiple increase of the DNA quantity.

Analyzed sample – biological material intended for research.

The internal control sample (internal control, IC) – DNA or RNA added to the analyzed sample in order to monitor the correct performance of all operations during the analysis. If there is no DNA or RNA in the sample, the internal control should be amplified (otherwise the result of the analysis is considered as invalid).

Histogram of optical measurements – graphical representation of the current values of optical measurements in three-dimensional coordinates.

Duplicate (duplicates) – a parallel set of two or several absolutely identical reactions. It is used to average the effect of random factors on the result.

Identificator – a unique designation of the reaction tube.

Intercalating dyes – substances that intensively fluoresce when bound to double-stranded DNA or RNA (eg, ethidium bromide, SYBR Green I).

Qualitative analysis – determination of the presence or absence of nucleic acids of the organism to be detected in the test sample.

Quantitative analysis – determination of the concentration of nucleic acids of the detected organism in the test sample.

Kinetics of DNA accumulation – graphical representation of the process of accumulation of DNA amplification products (amplicons).

Melting curve – a plot of fluorescence versus temperature upon heating (or cooling) the reaction mixture after the PCR termination.

Marker – numeric value of some parameter.

Matrix – certain arrangement of elements in space.

Multi-test mode – the option that allows combining several tests in one analysis with the same amplification program and analysis type.

Amplification program – a program that specifies the mode of carrying out the amplification reaction.

DNA or RNA dilutions – consecutive dilutions of DNA or RNA sample.

Fitting – smoothing of experimental data.

Fluorescent dye – a chemical compound capable of effectively fluorescing. Can absorb light energy of a certain wavelength (excitation wavelength) and re-emit it at another wavelength (wavelength of radiation).

Fluorophore – fluorescent dye.

Exposure of measurements – the parameter that regulates the sensitivity of the optical system of the device.

Appendix 2. Types of analysis (only the basic ones are represented)

When creating tests and working with different types of analysis, the settings of the analysis and device settings are requested from the kits manufacturer.

1.   Qualitative analysis

Qualitative analysis allows determining the presence or absence of the target DNA fragment in the sample.

To perform a qualitative analysis (Fig.99) it is necessary to select a **Qualitative analysis** from the **Type of analysis** drop-down list when creating a Test. In case of creation a plate protocol manually without using the **Test** option, this type of analysis is used by default.

In the first and second columns of the **Results** tab, the tube numbers and their identificators are indicated. The choice of the numbering type (by location in the thermal block or by the number in the plate protocol), as well as the display of the "color" of the tube, is made using the button **View data representation modes** below the table:

The results of a qualitative analysis are displayed in a column **Qualitative analysis** in the form of the following values:

+ – there are fragments of the desired DNA in the sample (the exponential growth of the signal for the *Specifics* channel is recorded).

- – there are no fragments of the desired DNA in the sample (there is no exponential signal growth for the Specific channel, in the case of using an internal control sample (IC), an exponential growth of the signal for the channel *IC*).

invalid – the unreliable result (when using IC - there is no exponential growth of the signal, both for *the Specifics* channel, and for the channel *IC*).
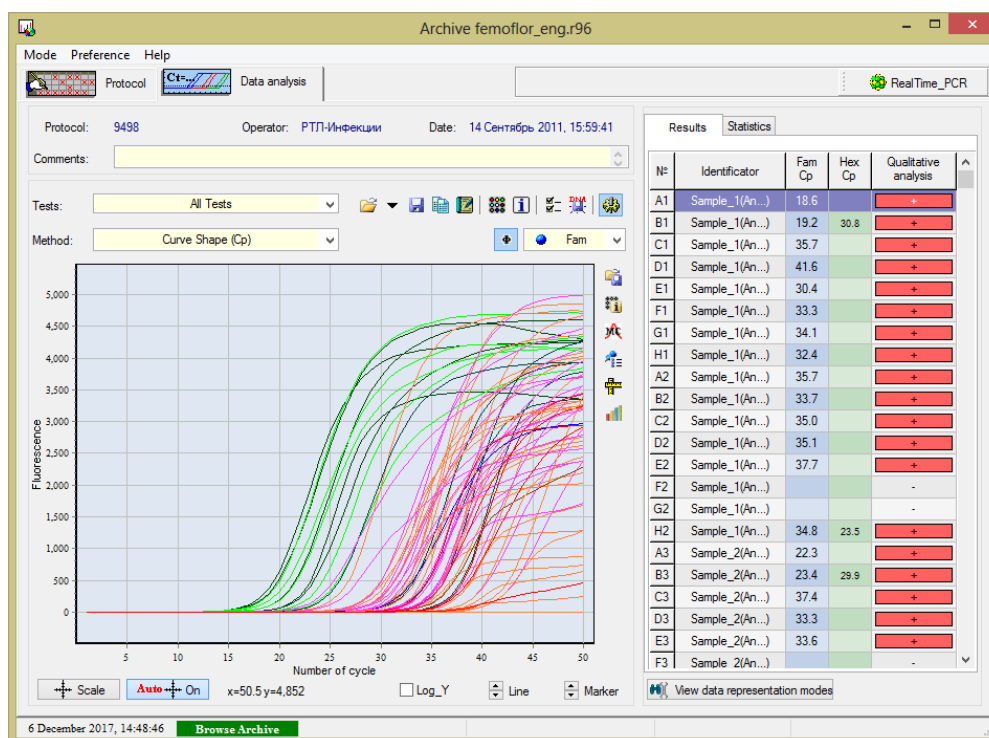
Fig.99 "Qualitative" type of analysis

In columns [Fam Ct] [Hex Ct] : the values of the output cycles for the fluorophores used for each sample (Ct or Cp – depending on the selected analysis method in the drop-down list **Method** of analysis).

2.   Quantitative with standards

Quantitative analysis using calibration samples ("standards") allows determining the amount of the desired DNA fragment in the sample. For the quantitative analysis, it is necessary to include the samples with a known amount of the desired DNA in the plate protocol (calibration samples, "standards").

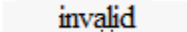There is option in the software that allows using calibration samples from other RUN file.

For quantitative analysis (Fig.100) it is necessary to select a **Quantitative analysis with standards** from the **Type of analysis** drop-down list when creating a Test.

After the analysis, if there are calibration samples, the software will automatically build a calibration plot (you can click the button to view it **Show the "Standard curve" plot in the basic toolbar "Panel of device "**) and will determine the concentration of the desired DNA in unknown samples.
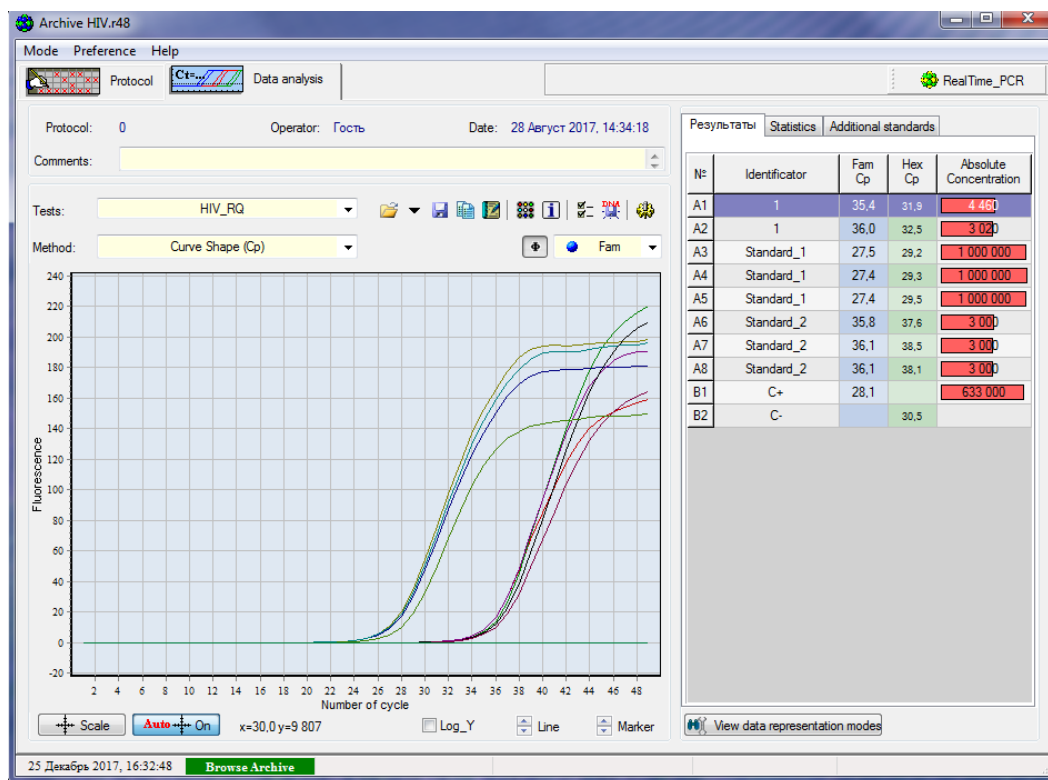
Fig.100 "Quantitative with standards" type of analysis

When using option of adding the calibration samples from other RUN file, it is necessary to perform the following actions:

–   proceed to the tab **Additional standards** in the **Archive** mode window;

–   click the button , select the file with the corresponding RUN (file type *.r48, *.r96, *.384), the "standards" of which will be used as additional standards. For detailed information see **Additional standards**.

The results of the quantitative analysis are displayed in a column **Absolute concentration**.

In columns ![Fam Ct | Hex Ct]: the values of the "characteristic" cycles for the fluorophores used for each sample, (Ct or Cp – depending on the selected analysis method in the drop-down list **Method** of analysis).

Interpretation of the results of the analysis is carried out in accordance with the instruction for use to the PCR kit used.

❖ **Additional standards**

For analysis, it is possible to use "standards" (samples with the known content of initial concentration) from another RUN file or **Additional Standards** (Fig.101).

In this case, there is option to conduct analysis in one run up to 96 tubes with unknown samples.
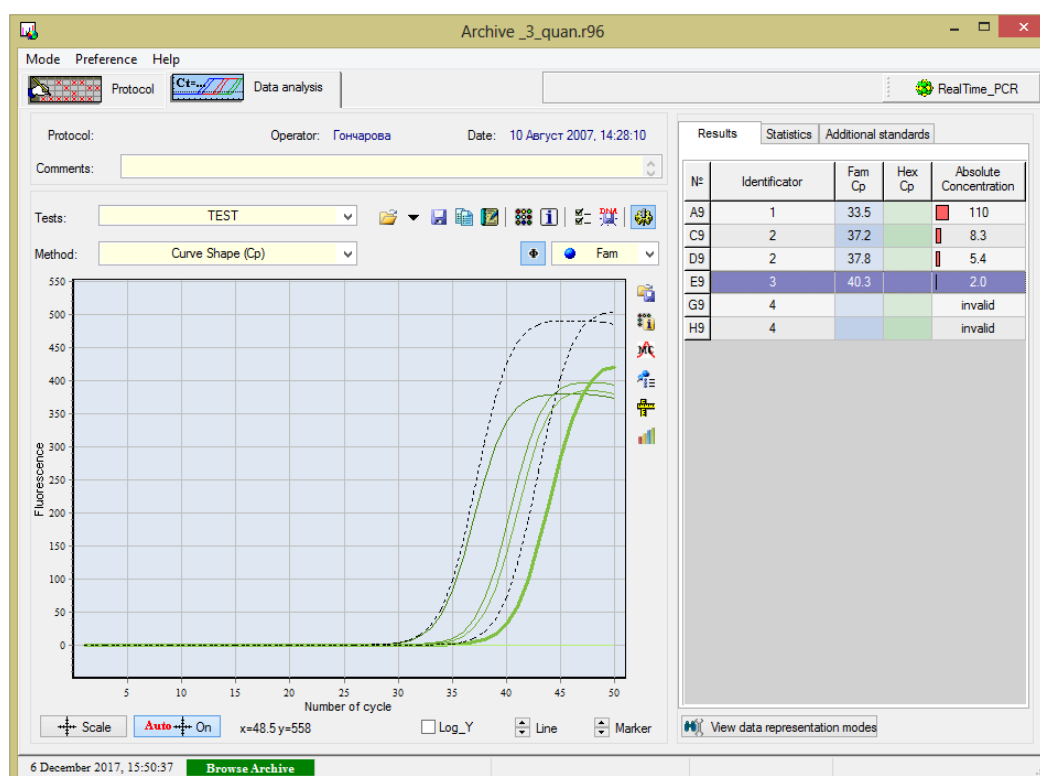
Fig.101 Display of additional standards when selecting the type of analysis "Quantitative with standards"

Attention! An obligatory condition for the use of additional standards is the identity of the RUN (amplification program, test type).

Additional standards can be added:

- at the stage of creating a plate protocol using the procedure **Add test**;
- at the stage of the results analysis in the tab **Additional standards**.

Information about additional standards is displayed in the tab **Additional standards**.

❖ **Standard curve**

The following data is displayed in the **Standard curve** window (see Fig.102):

- coordinate system (X-axis is the logarithm of the sample concentration **Log10 (Concentration)**, the Y-axis is the value of Ct or Cp);
- calibration samples (indicated by ⬤);
- studied samples (indicated by ▯);
- "standard curve";
- the efficiency of PCR, the standard deviation, the reliability of the approximation.
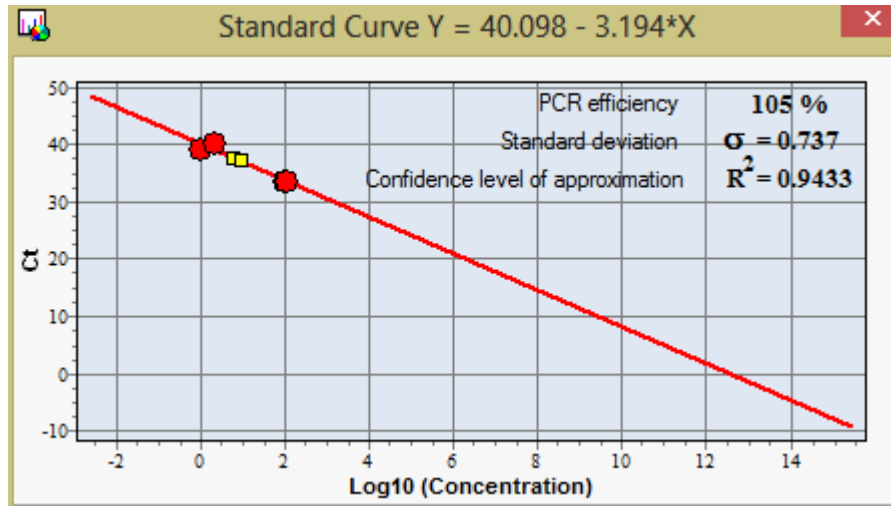

Fig.102 The window Standard curve when selecting the type of analysis "Quantitative with standards"

Note! The software constructs straight line of the form $Y = A*x+B$ from the points of the calibration samples ⬤, for which the concentration and the value of Ct (or Cp) are known**.** The equation of the resulting straight line is indicated in the name of the **Standard curve window**.

On the constructed straight line the investigated samples are displayed in the form of icons ▯, in accordance with the obtained value of Ct (Cp). Based on the Log10 concentration found, the software calculates the concentration of the analyzed samples.

The quality of the approximation of the "Standard curve" is represented by the **Confidence level of approximation** and the **Standard deviation** values.

3. Relative analysis

The Relative analysis makes it possible to estimate the amount of the desired DNA fragment in the test samples relative to the "base" tube. The value of the relative concentration of the "base" tube is one. When conducting a relative analysis, it is assumed that the efficiency of PCR for all samples is the same. Any of the samples can be selected as a "base".

To carry out a relative analysis (Fig.103), select a **Relative analysis** from the **Type of analysis** drop-down list when creating a Test.

To select a base sample, proceed following:

- open the window **Relative concentration**, located in the upper left corner of the fluorescence graph (click the icon ▢);
- in the opened **Relative concentration** window choose one of the following values:

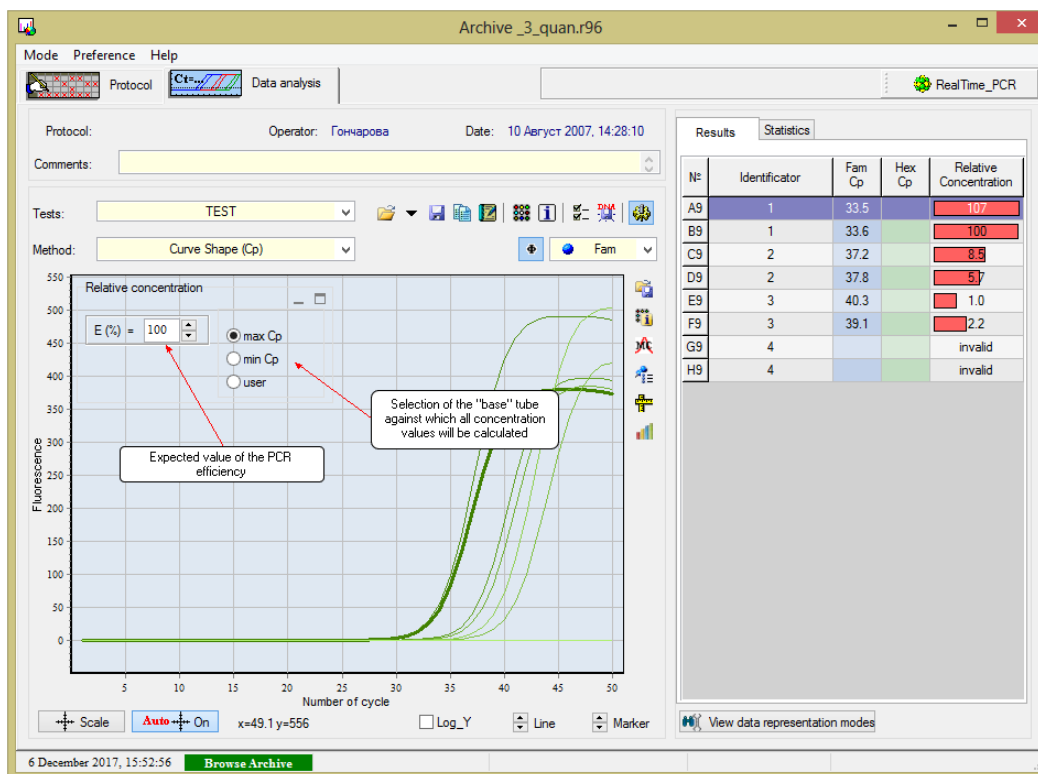⊙ max Ct – a sample with a maximum value of Ct (or Cp),

Fig.103 "Relative" type of analysis

When the option **User** is enabled, two additional lists appear:

A1 – selection of the base test tube,

Fam – selection of the optical measurement channel for the base tube.

By default, the relative concentration is calculated assuming a 100% efficiency of the PCR reaction.

If the titration indicators of the individual samples are known, the result of the values of the relative concentration can be corrected by changing the value of the efficiency coefficient using the field E [%] = 100 .

The results of the relative analysis are displayed in a column **Relative concentration** as the relative concentration of the amplified fragments (amplicons) in each of the tubes relative to the tube chosen as the base one.

In columns Fam Ct | Hex Ct : the values of the "characteristic" cycles for the fluorophores used for each sample (Ct or Cp – depending on the selected analysis method in the drop-down list **Method** of analysis).

4. Multiplex detection

Multiplex detection in this section means the types of data representation. For all RUNs in which classical PCR is present, it is possible to select the type of presentation **Multiplex detection** from the

types of analysis (Fig.104). Information on all channels in each tube shows information of a qualitative, quantitative or relative analysis, respectively. This type of presentation is designed to work in multi-channel mode, and it allows detecting up to five specific DNA fragments simultaneously.

Note! To conduct a relative analysis, select a **Multiplex detection** from the **Type of analysis** drop-down list when creating a Test.
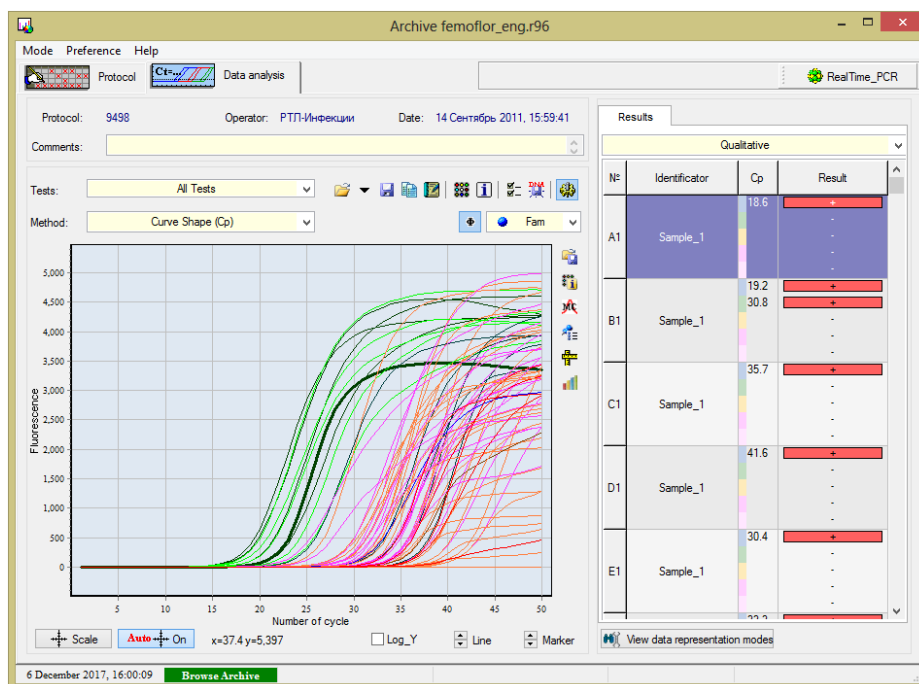
Fig.104 Type of representation «Multiplex **detection**»

5. Melting curve

For all RUNs in which the procedure of "melting" of probes is present, select the **Melting curve** type of data representation from the corresponding item of analysis types that allows estimating the values of the temperature melting peaks for future analysis of genetic polymorphisms (Fig.105).

Note! To conduct a **Melting curve (MC)** analysis, select the related **Analysis of polymorphism (MC)** from the **Type of analysis** drop-down list when creating a Test.
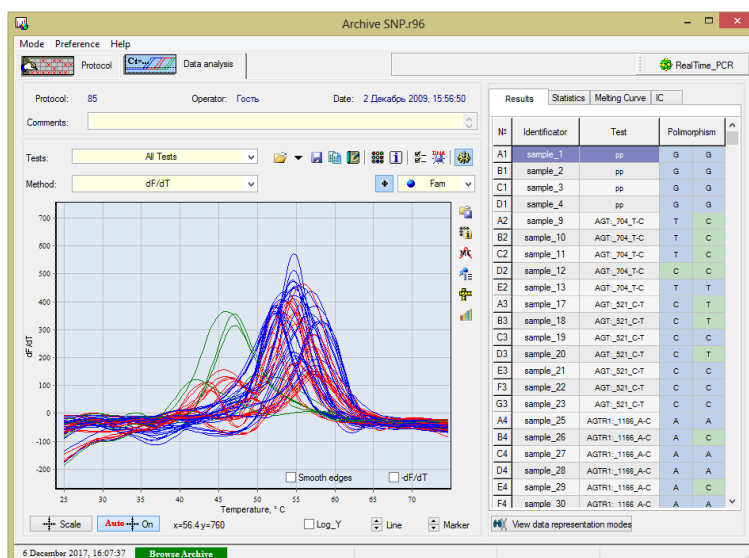

Fig.105 Type of representation "Melting curve"

Appendix 3 DTReport library description

The DTReport library is intended for implementation of algorithms' analysis and results' calculation based on data obtained from RealTime_PCR software.
The library includes the following components:
- DTReport core;
- a file with code on the DTReport computation language ("dtr file");
- a file with PCR test parameters («ini-file»);
- report generator.

The DTReport library provides wide opportunities for assay calculations and for visualizing the calculated results in the form of tables and reports.
1. Computing modules
Any type of analysis is implemented by means of a calculating module (hereinafter referred to as a module) developed in the DTReport Studio environment (application is not included in the library). The module contains the following components:
- calculation of results;
- generating reports;
- visualization of results;
- settings of assay parameters.

The module is built into the application using the DTReport core. Such architecture allows using of the same compute modules with RealTime 7 software.
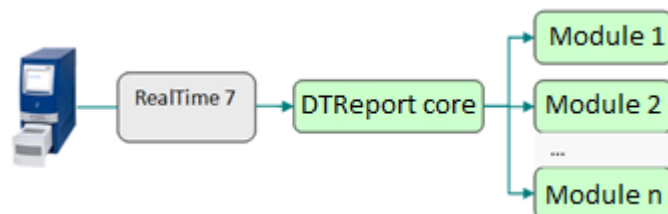

Fig. 106 Architecture of interaction between RealTime_PCR software, DTReport core and computational modules

2. Data structure
DTReport operates with data organized in a hierarchical structure of the following structure:
Document (protocol)
          Request
               Sample
                    Test
                         Result

Calculated elements can be added to each level of this hierarchy.

```
-<Inquiry ID="0" Date="20 Апрель 2018, 10:51:49">
  -<Sample ID="0" Code="Образец_1">
    -<Service ID="#0.21" ServiceID="21" Name="Fomoflor Screen2.0">
        <DTReport FormID="Femoflor"> </DTReport>
        <Result OriginalValue=" 18.878011861" TestID="#Tube1.ОБМ" ValueName="Cp" Value=" 18.878011861" Name="ОБМ" ProgName="TBM"
```

3. Localization
DTReport Studio includes localization tools that allow to localize both the system core and the computing modules without rebuilding the software, including:
- report form;
- string constants used in calculations;
- GUI elements (settings of the results' presentation).

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