

Thermo Scientific PikoReal Software 2.1

User Manual

Rev. 2.1



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User Manual

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About This User Manual

Intended users

PikoReal Software is intended to be used in conjunction with Thermo Scientific PikoReal Real-Time PCR Systems in research laboratories by professional personnel.

How to use this user manual

This user manual covers the use of PikoReal Software in conjunction with the following instruments: 24-well PikoReal Real-Time PCR System (Cat. no. TCR0024) and 96-well PikoReal Real-Time PCR System (Cat. no. TCR0096). It has been designed to give you the information you need to:

- Install PikoReal Software
- Operate the software
- Adjust the software settings

For information on using the PikoReal Real-Time PCR System, refer to the *Thermo Scientific PikoReal Real-Time PCR System User Manual* (Cat. no. N11469). This manual assumes familiarity with the instrument user manual.

Read the manual in its entirety before using the software.

Keep the user manual for future reference. The user manual is an important part of the software and should be readily available.

For more information

For the latest information on products and services, visit our website at:

<http://www.thermoscientific.com/pikoreal>

In our efforts to produce useful and appropriate documentation, we would appreciate any comments you may have on this user manual for your local Thermo Fisher Scientific representative.

About This User Manual

Contents

Intended users	3
How to use this user manual	3
For more information	3
PikoReal Software.....	9
Installing PikoReal Software	11
Recommended PC setup	11
Starting the installation	11
Connecting instruments	12
Instrument IP address	13
Instrument name and location	13
User Interface	15
Starting PikoReal Software	15
Navigating the software	16
Home view	17
View selection	19
Protocols.....	21
Creating a protocol	22
Protocol properties	24
Fluorophores	24
Reaction volume	24
Advanced properties	24
Cycle step	25
Temperature step	25
Data acquisition	27
Channel wavelengths	27
Melt Curve step	27
Importing and exporting protocols	28
Plate Layout.....	29
Creating a layout	30
Sample type	32
Replicates and quantity series	33
Grouping samples	33
Importing and exporting layouts	34
Executing an Experiment	35
Executing an existing experiment	36
USB protocol transfer	36

Monitoring a Run	37
Adding reagents during a run	38
Adding cycles	38
Protocol status.....	39
Analysis	41
Run log	42
Adding analyses.....	42
Analyzing sample groups	43
Exporting analyses.....	43
Cq and Absolute Quantification	45
Adding an absolute quantification analysis	46
Graphs	46
Graph views	47
Settings	48
Baseline.....	48
Cq methods	49
Individual Cq.....	49
Threshold.....	50
Importing a standard curve	50
Results	51
Relative Quantification	53
Adding a relative quantification analysis.....	55
Graph views	55
Amplification efficiency.....	56
Settings	56
Results	57
Allelic Discrimination	59
Layout creation for allelic discrimination.....	60
Adding an allelic discrimination analysis	60
Graph views	61
Settings	62
Virtual standards.....	62
Manual call	62
Melt Curve	63
Adding a melt curve analysis	64
Graph views	65
Settings	65
Peaks.....	66
Results	66
High-resolution Melt Analysis	67
Adding an HRM analysis	68
HRM analysis and results.....	68

Graph views	69
Settings	69
Settings	71
Well colors	72
Fluorophores	72
Instruments	73
Color Calibration	75

Chapter 1

PikoReal Software

PikoReal Software is a practical PC software application for setting up, managing and running PikoReal Real-Time PCR Systems in the laboratory.

PikoReal Software is used to:

- Set up and configure your PikoReal instruments
- Define and run protocols on your PikoReal instruments
- Store and analyze instrument runs

PikoReal Software provides the following analysis methods:

- Absolute quantification
- Relative quantification
- Allelic discrimination
- Melt curve analysis
- High-resolution melt analysis

Chapter 2

Installing PikoReal Software

This chapter describes the installation of PikoReal Software.

Recommended PC setup

The following table details the recommended PC setup for use with PikoReal Software.

Table 2–1. Recommended system requirements

Recommended setup	
Supported operating systems	Microsoft Windows 7 Microsoft Windows XP Professional with Service Pack 3 or later
Disk space requirement	100 MB
Processor recommendation	Dual-core processor
Memory recommendation	4 GB of RAM
Network connection requirement	Ethernet connection for instrument
Monitor resolution requirement	1280 x 960 resolution (minimum)

For information on installing PikoReal Software on to a Mac, refer to document *Installing Thermo Scientific PC Software on to Mac OS* (Cat. no. D11362).



Note If you are upgrading from PikoReal Software 2.0, there is no need to remove the previous software version. The installer application performs the necessary actions. ▲

Starting the installation

The installation of PikoReal Software should begin automatically once you insert the software CD into your computer's CD-ROM drive. If the installer does not start automatically, you can launch it by double-clicking the installer file (*Setup.exe*) on the software CD.

The installer launches and lists the software components to be installed. Note that Microsoft .NET Framework 4.0 automatically installed, if it is not detected on the computer.

Click *Install* to begin the installation.

Follow the instructions presented by the installer to complete the installation of PikoReal Software.

After installation, you can start PikoReal Software by double-clicking the PikoReal Software icon on your desktop.

Connecting instruments

PikoReal instruments are connected to the host PC via a direct Ethernet network connection. No more than five instruments should be connected to PikoReal Software simultaneously. Connecting instruments to a computer via an office network is not recommended.

PikoReal Software detects available instruments automatically, if the required network settings are correct, and the instruments are switched on and connected to the computer via an Ethernet connection.



Note You need to connect instruments to the PC either directly or via a network hub or switch. This will probably require you to disconnect your computer's network connection. If a connection to an external network is required, you can accomplish this, for example, by using a USB-to-Ethernet adapter. ▲

To modify the host computer's network settings to accommodate a direct instrument connection:

1. Connect an Ethernet cable directly between the instrument and PC.
2. With Windows 7, in the *Windows Control Panel*,
 - Select Network and Internet > Network and Sharing Center > **Change Adapter Settings**
 - Right-click *Local Area Connection* and select **Properties** from the context menu
 - Select **Internet Protocol Version 4 (TCP/IPv4)** from the list and click the **Properties** button.

With Windows XP, in the *Windows Control Panel*,

- Select **Network Connections**.
 - Open the active *Local Area Connection*.
 - On the *General* tab, select **Internet Protocol (TCP/IP)** from the list and click the **Properties** button.
3. Select *Use the following IP address*. Enter 192.168.007.220 as the *IP address* and 255.255.255.0 as the *Subnet mask*.
 4. Click **OK** followed by **OK** to confirm the changes.

See “Instrument IP address” on page 13 for information on changing the IP address of a connected instrument.



Note Other network configurations are possible. Contact your network administrator for assistance. For firewall configurations, PikoReal Software uses TCP port 62751 to communicate with instruments. ▲

Instrument IP address

The default IP address of a new PikoReal instrument is 192.168.007.221. If several instruments are connected to the same computer, they must have unique IP addresses. It may, therefore, be necessary to modify the IP addresses of connected instruments.

To change the IP address of an instrument:

1. Click the **Settings** button in the **Home** view to open PikoReal Software settings.
2. Select the **Instruments** page.
3. Select the instrument from the list and enter the new IP address into the *IP Address* field.
4. Click **Update** to confirm the change and send the information to the instrument.

It may take several minutes for the updated instrument to reappear in the instrument list.



Note The first three digit sets of the instrument's IP address must be identical to the IP address of the host computer, and the final digit set must be a unique number. Avoid using 000 in any digit set. The final fourth digit set must be between 002 and 254 excluding 220, which is reserved for the PC. ▲

Instrument name and location

To identify several connected instruments, you can assign them a name and a location. The given instrument name is also displayed on the instrument display.

To define a name and location for an instrument:

1. Click the **Settings** button in the **Home** view to open PikoReal Software settings.
2. Select the **Instruments** page.
3. Select the instrument from the list and enter the name and the location into the corresponding fields.
4. Click **Update** to confirm the changes and send the information to the instrument.

Chapter 3

User Interface

This chapter describes the user interface and general use of PikoReal Software.

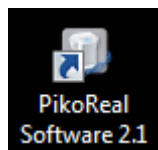


Starting PikoReal Software

Launch PikoReal Software from the Start menu:

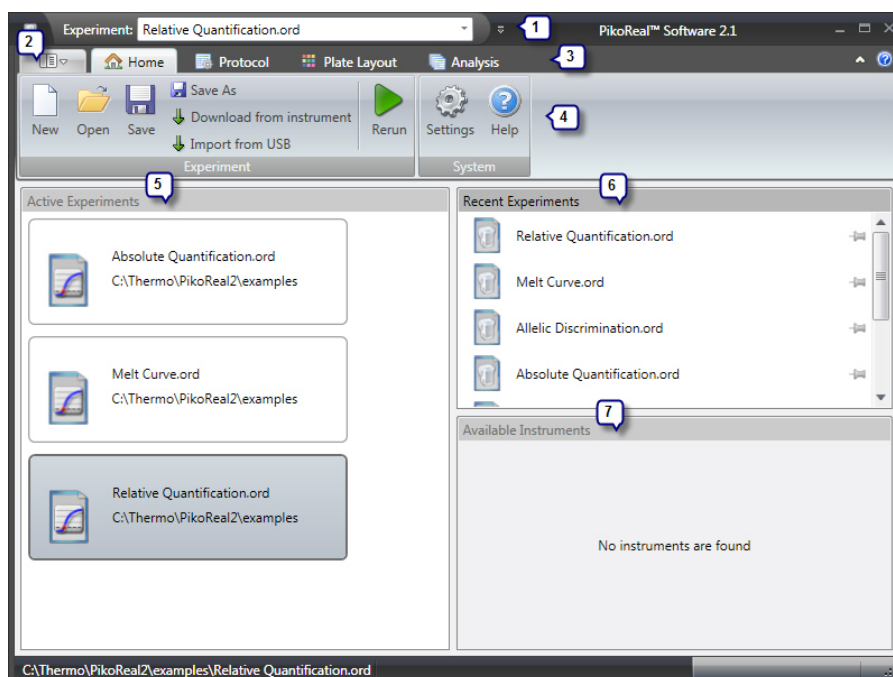
Start > All Programs > Thermo PikoReal Software 2.1 > PikoReal Software 2.1,

or by double-clicking the desktop icon.



Navigating the software

Launching PikoReal Software opens the application's main window with the **Home** view.



The main application window contains the following elements:

- *Title bar* (1)
Save button and active experiment menu.
- *Application menu* (2)
Quick access from any view to the following commands: *New*, *Open*, *Save*, *Save As*, *Settings*, *Help*, *About* and *Exit*.
- *Navigation bar* (3)
Tabs for selecting the view. Only the currently selectable tabs are shown.
- *Action panel* (4)
View-specific function buttons.
- *Active Experiments* (5)
Experiments that are currently loaded into memory. To use an experiment select it by clicking.
- *Recent Experiments* (6)
A user-definable list of recently used experiments. Double-click an experiment to load it and make it active. To keep an experiment on the list, click the pin icon next to the experiment.
- *Available Instruments* (7)
A list of the available instruments on the network.

When you launch PikoReal Software, only the **Home** view is available. After you create a new experiment or reopen an old experiment, the **Protocol** and **Plate Layout** views are available. The **Current Status** view

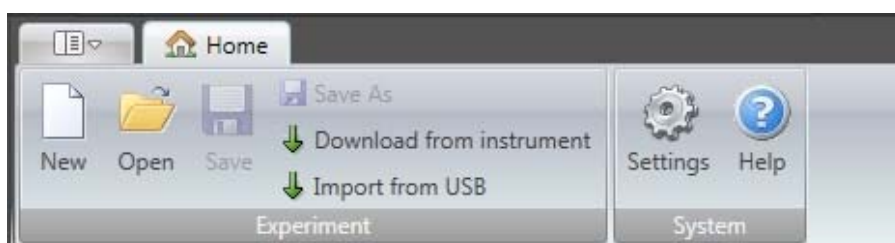
is shown while an experiment is running and the **Analysis** view is available after an experiment has been completed. The window contents depends on the selected view. Buttons and controls are active only when they can be used.

Home view

The **Home** view opens when you launch PikoReal Software. In the **Home** view, you can create new experiments, open existing experiments, and save newly created and modified experiments.

You can also import experiments from an instrument on the network or from a connected USB memory device.

When you open PikoReal Software without active experiments, only the **Home** view is available with the *Experiment* and *System* action panels.



When you create a new experiment, the **Protocol** and **Layout** tabs appear and the **Run** button is available in the *Experiment* action panel.


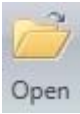
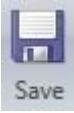
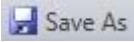



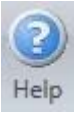



When you open experiments that have been run, the **Analysis** tab appears and the **Run** button changes to the **Rerun** button.



The **Home** view action panel buttons are described below.

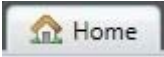
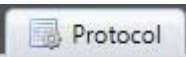

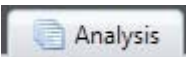
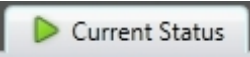
Table 3–2. Home view action buttons

Button	Description
	Create a new experiment.
	Open an existing experiment.
	Save the current experiment.
	Save the current experiment with a new name.
 Download from instrument	Retrieve the data from an instrument. Instruments retain run data from the latest run.
 Import from USB	Import a protocol run from a USB memory device. Refer to the instrument’s user manual on transferring run data to a USB memory device.
	Open PikoReal Software settings.
	Open PikoReal Software help.
	Start or rerun the current experiment.

View selection

Select the desired view with the navigation tabs at the top of the main window. Availability of the tabs depends on the status of the software.

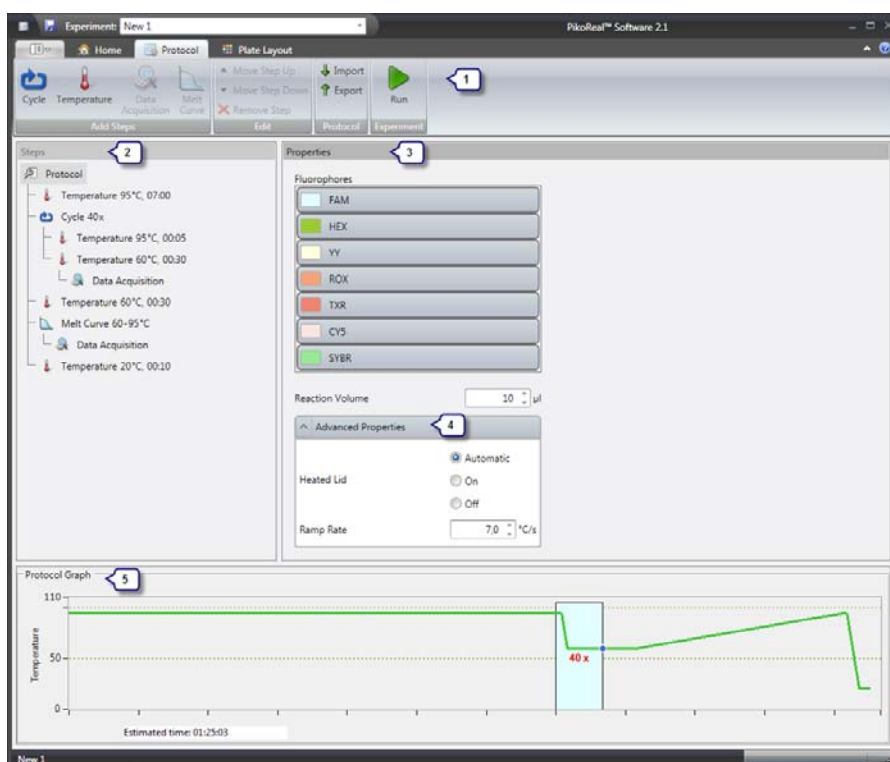
Table 3–3. Application views

Tab	Available functions
 Home	Create, open and save experiments. Import experiments from instruments or USB memory devices. Access PikoReal Software settings and help. Start the protocol or rerun the current experiment.
 Protocol	Create or edit the protocol steps of the current experiment. Import and export protocol files. Start the protocol or rerun the current experiment.
 Plate Layout	Define or edit the sample layout of the current experiment. Manage replicates and sample groups. Select the plate type. Import and export layout files.
 Analysis	Add analyses to the current experiment. Edit the analyses of the current experiment. Export experiment data as a Microsoft® Excel® report or as raw data.
 Current Status	Shown only when an experiment is running. View the fluorescence data in real time. Open and close the instrument drawer. Skip and add cycles to a step. Abort protocol execution.

Chapter 4

Protocols

A protocol is a sequence of thermal cycling and data collection instructions given to a connected instrument. In the **Protocol** view, you can create and edit the protocol of the current experiment.



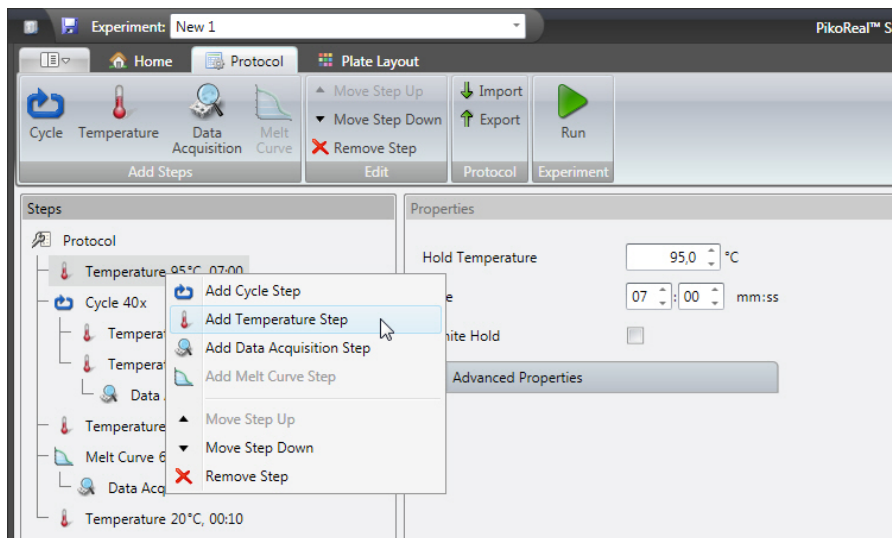
The **Protocol** view contains the following main fields:

- *Action panel* (1)
Action buttons for adding, removing and reordering steps, importing and exporting protocols, and running the experiment.
- *Protocol steps* (2)
Step sequence tree for viewing and selecting steps for editing. Click a step to view and edit its properties.
- *Step properties* (3)
Properties of the selected protocol step.
- *Advanced step properties* (4)
Advanced properties of the selected protocol step.

- *Protocol graph (5)*
Protocol temperature graph with the run-time estimate.

Creating a protocol





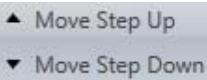
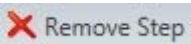
You can create a new protocol by editing the default protocol or an existing protocol. To open an existing protocol for editing, import a protocol file (.ptp) by clicking the **Import** button in the **Protocol** view.



Edit the protocol by first selecting a protocol step and then using the buttons in the *Steps* and *Edit* action panels, or by right-clicking a step and selecting the command from the context menu.

You can also drag and drop protocol steps to move them in the step sequence.

Table 4–4. Protocol editing commands

Button	Function	Description
	Add Cycle Step	Adds a cycle step, which can include temperature steps. Select the cycle step and click the Temperature button to add temperature steps to the cycle.
	Add Temperature Step	Adds a temperature step, for which you can define the temperature and time, as well as set an infinite hold and define advanced properties.
	Add Data Acquisition	Adds data acquisition to a temperature step.
	Add Melting Curve Step	Adds a melting curve step, for which you can set the start and end temperatures, as well as advanced properties.
	Move Step Up/Down	Moves the selected step upwards or downwards within the protocol.
	Delete Step	Removes the selected step from the protocol.

Click the **Cycle**, **Temperature**, or **Melt Curve** button to add the corresponding step to the protocol. The new step is added below the selected step.

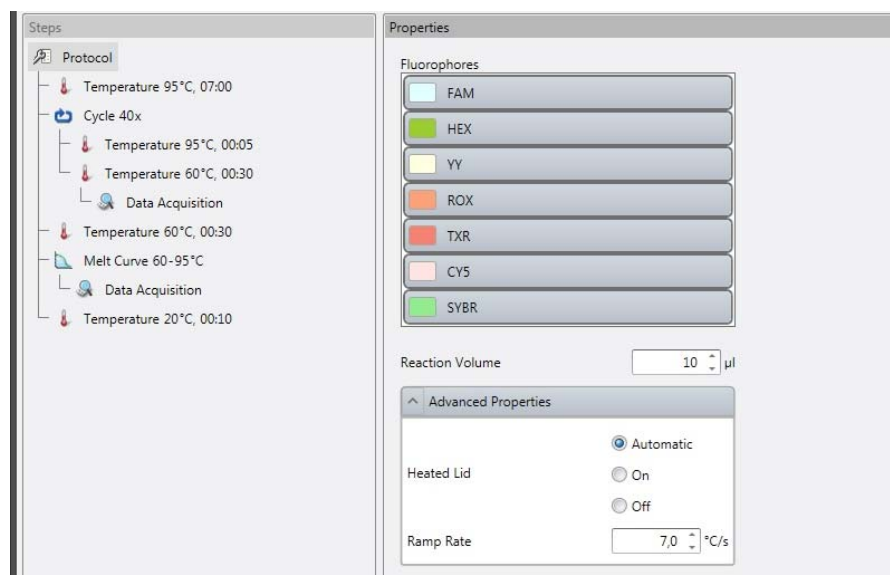
Click the **Data Acquisition** button to add data acquisition to the selected temperature step. The melting curve step contains data acquisition by default.

The protocol is executed as a sequence from top to bottom, that is, the first step is at the top and the last is at the bottom. You can change the execution order of the steps in a protocol by selecting a step and clicking the **Move Step Up** or **Move Step Down** buttons.

Delete steps from the protocol by highlighting them and clicking the **Remove Step** button.

Protocol properties

Select the Protocol icon in the protocol sequence to view the *Protocol Properties* and define the *Fluorophores* and *Reaction Volume* used.



Fluorophores

All fluorophores are selected by default in a new experiment. Click a fluorophore to disable or enable it. You can define the colors used to designate the fluorophores in *Settings > Fluorophores*.

We recommend that you select only the fluorophores that are required for the experiment.



Note Only the selected fluorophores and corresponding channels are measured and available in the layout. Unmeasured channels cannot be added after an experiment has been run. ▲

Reaction volume

Enter the reaction volume. The range is 10–50 µl for the 24-well instrument and 5–20 µl for the 96-well instrument.

Extended hold time is recommended for 20 µl sample volumes with the 96-well instrument.

Advanced properties

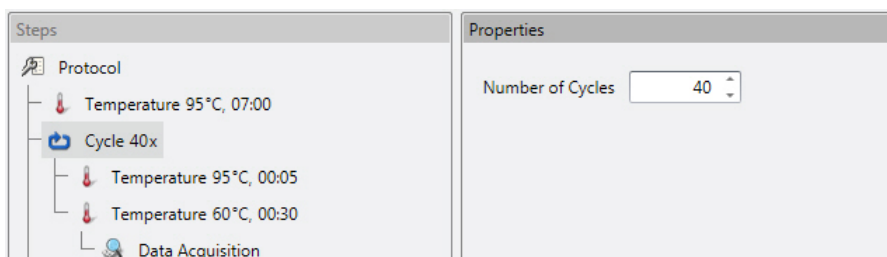
Click the **Advanced Properties** button to:

- Control the *Heated Lid*. Choose *Automatic* to allow the system to automatically select the temperature based on the reaction volume and the protocol. Select *On* to manually set the lid temperature or *Off* to disable the lid heater.
- Define the *Ramp Rate*, which is the steepness of the temperature ramp. The value is defined as degrees Celsius per second and

the range is 0.1–7.0°C/s. Individual ramp rate settings override this default setting. See “Temperature Step” on page 25.

Cycle step

Add a cycle step to a protocol by clicking the **Cycle** button, or by right-clicking the protocol step sequence and selecting **Add Cycle Step** from the menu. The new empty cycle step is added below the currently selected step.



A cycle step can contain one or several temperature steps with or without data acquisition.

Add temperature steps to a cycle step by selecting the cycle step and clicking the **Temperature** button, or by right-clicking the cycle step and selecting **Add Temperature Step** from the menu. You can also move existing temperature steps to a cycle step by clicking and dragging them. When you move a cycle step, all of its contents moves with it.

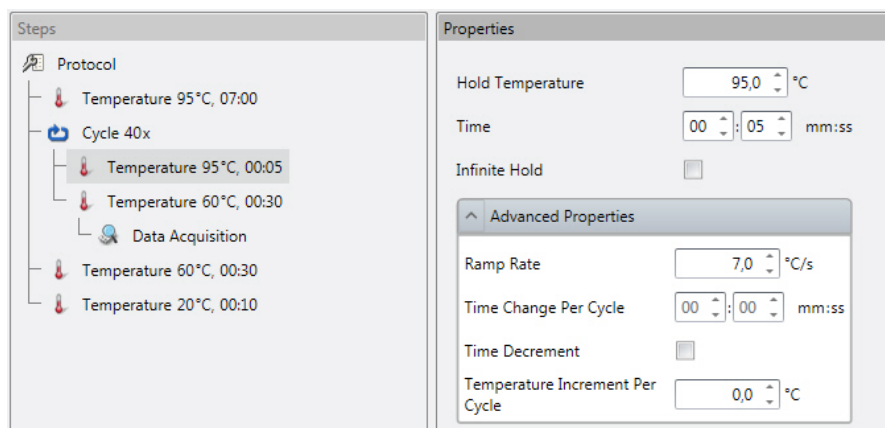
Use the *Number of Cycles* property to set the number of cycles in the selected step.

To collect data, select *Data Acquisition* for each of the required steps.

Temperature step

Add a temperature step to a protocol by clicking the **Temperature** button, or by right-clicking the protocol step sequence and selecting **Add Temperature Step** from the menu. The new temperature step is added below the currently selected step.

If you wish to include the new temperature step in a cycle, select the cycle step before adding the temperature step or click and drag the new temperature step to the cycle step.



After adding a temperature step,

1. Define the target temperature using the *Hold Temperature* control. The temperature range is +4.0–99.9°C.
2. Set the step duration using the *Time* control. The duration range is 00:00–59:59 (mm:ss).

OR

Select *Infinite Hold*.

Infinite Hold maintains the defined temperature until the user confirms protocol continuation by clicking **Next** in the **Current status** view.

3. Add *Data Acquisition*, if you wish to acquire fluorescence data at this point in the protocol. Data is collected at the end of the step to which it is assigned.

In addition to the basic settings, you can configure the following temperature step properties by clicking the **Advanced Properties** button.

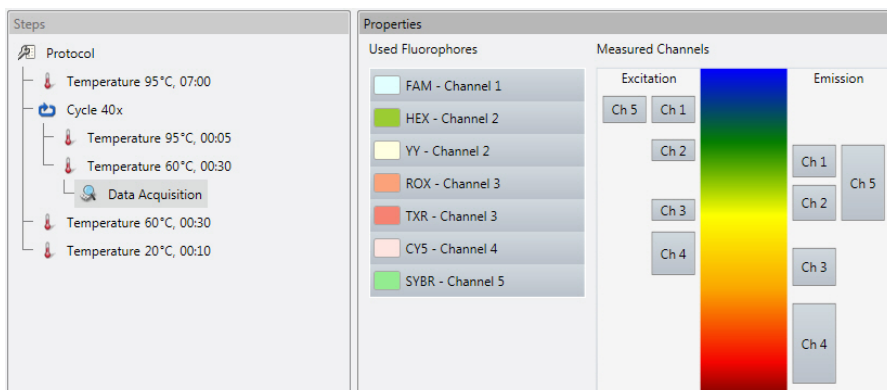
Ramp Rate defines the speed of the temperature ramp between individual temperature steps, to achieve the selected hold temperature. The value is defined as degrees Celsius per second and the range is 0.1–7.0°C/s. Individual ramp rate settings override the default ramp rate defined in the *Protocol Properties*. See “Protocol properties” on page 24.

Time Change Per Cycle allows you to define a fixed time increment (or decrement) per cycle until a time range limit is reached for a temperature step within a cycle step. To decrease time, tick the *Time Decrement* box.

Temperature Increment Per Cycle allows you to define a fixed temperature increment per cycle until a temperature range limit is reached for a temperature step within a cycle step. Note that the temperature increment can also be negative.

Data acquisition

Data acquisition can be added to an individual or cycled temperature step. A melting curve step contains data acquisition by default.



Select the **Data Acquisition** substep to view its properties, which present the channel and fluorophore mapping and the channels that have been selected for measurement.

Channel wavelengths

The detection channel wavelength ranges of the PikoReal instrument are presented below.

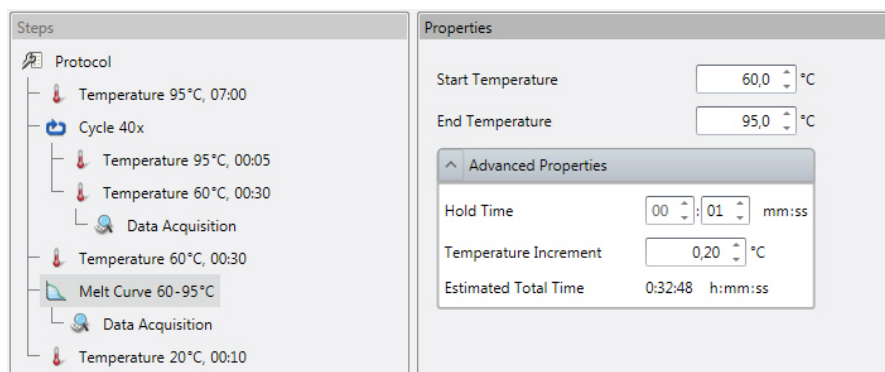
Table 4–5. Detection channel wavelength ranges

Detection channel	Excitation wavelengths	Emission wavelengths	Fluorophore
1	475–500 nm	520–550 nm	FAM
2	515–535 nm	557–590 nm	HEX, Yakima Yellow
3	570–590 nm	615–650 nm	ROX, Texas Red
4	600–640 nm	666–740 nm	CY5
5	475–500 nm	520–590 nm	SYBR Green

Melt Curve step

Add a melt curve step to a protocol by clicking the **Melt Curve** button, or by right-clicking the protocol step sequence and selecting **Add Melt Curve Step** from the menu. The new melt curve step is added under the currently selected step.

Note that there can be only one melt curve step in a protocol and that it cannot be placed within a cycle step.



Use the *Start Temperature* and *End Temperature* controls to set the beginning and end temperatures of the melt.

Click the **Advanced Properties** button:

- *Hold Time* defines the duration of one step, and can be set to between 00:00 and 59:59 (mm:ss).
- *Temperature Increment* defines the increase in temperature between steps, and can be set to between 0.04–5.00°C.
- *Estimated Total Time* is an estimate of the time required to complete the melt curve step.



Note The minimum actual hold time depends on the number of channels measured and may be as high as 13 seconds even when the *Hold Time* setting is set to 00:00. ▲

Importing and exporting protocols

You can import and export protocols as separate files with the *.ptp* file extension. This allows you to handle protocols separately from experiments. Note that you cannot import protocols directly from an experiment.

To import a protocol file, click the **Import** button in the *Protocol* action panel of the **Protocol** view. Browse to the file and click **Open**.

To export a protocol file, click the **Export** button in the *Protocol* action panel of the **Protocol** view. Browse to the desired file location, name the protocol file and click **Save**.

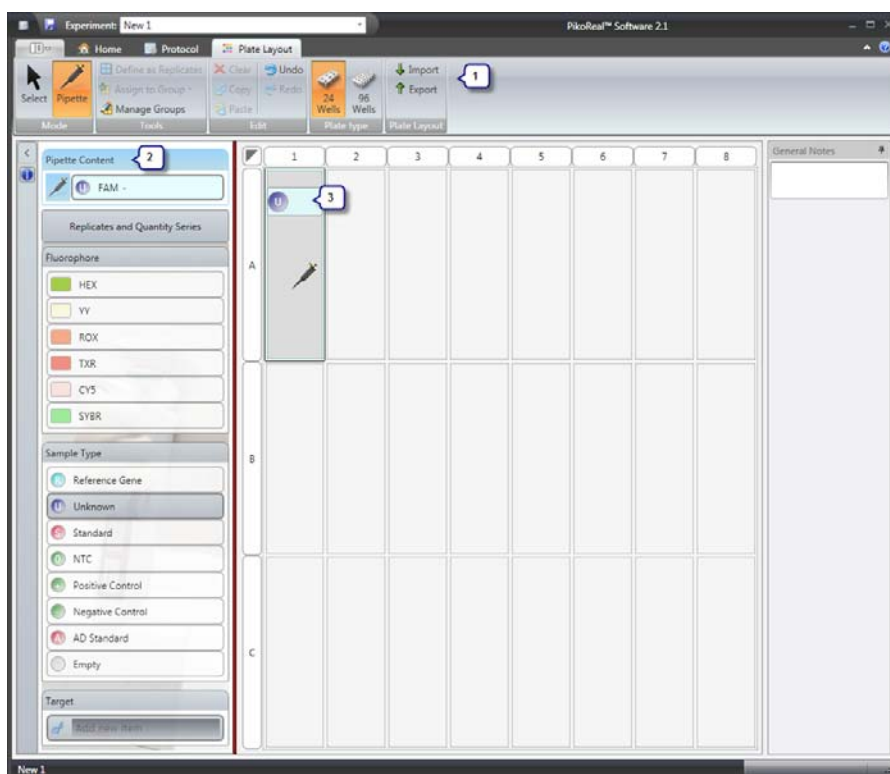
Chapter 5

Plate Layout

Plate layout refers to the location of samples in the plate wells. The layout contains, for each plate well, information on the fluorophore, target, sample type, and quantity of a standard as well as descriptive information.

You can create and edit the layout in the **Plate Layout** view.

The Virtual Pipetting Tool™ assists in entering the data into the plate quickly and in a manner resembling reagent pipetting in the laboratory. This intuitive tool guides you through defining the plate layout with ease and visualizes the information entered.



The **Plate Layout** view contains the following fields:

- *Action panel (1)*
Action buttons for entering well content and sample properties using the *Pipette* and *Select* modes, creating replicates and sample groups and importing and exporting layout files.
- *Pipetting panel/Editing panel (2)*
Depending on the selected mode (*Pipette* or *Select*), this panel is

used to pipette well contents, fill quantity series and replicate data, or edit sample properties.

- *Plate layout (3)*
Graphical representation of the contents of each well.

The layout can be empty when running a protocol. An edited layout can also be modified during or after protocol execution.

Creating a layout

You can create a new plate layout from a blank template or by editing an existing layout. To edit an existing plate layout, import the layout from a layout file using the **Import** button in the *Plate Layout* action panel.

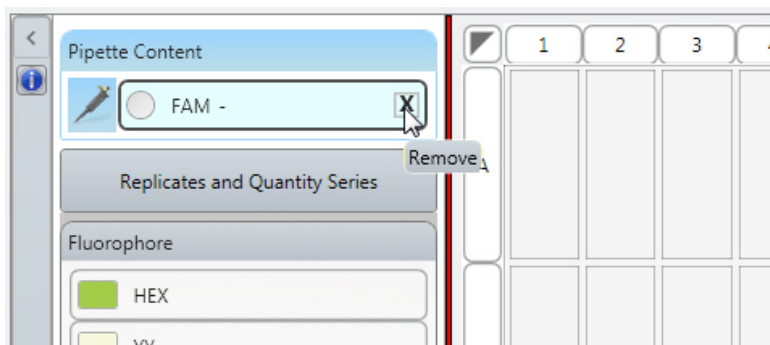
The **Plate Layout** view has two modes: *Pipette* and *Select*. The *Pipette* mode is used to select fluorophores and sample types into the *Virtual Pipette Tool* and to fill the plate wells. The *Select* mode is used to define sample properties and add descriptions as well as to create sample groups. The mode is selected by clicking the appropriate button in the *Mode* action panel.



Note Fluorophores must be pipetted into the layout before the corresponding sample properties can be defined. ▲

To create a plate layout:

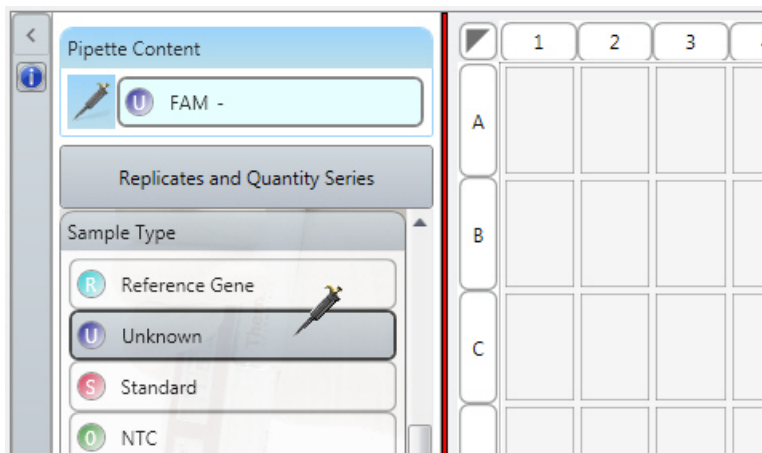
1. Click the **Pipette** button in the *Mode* action panel to change to the pipetting mode in the **Plate Layout** view.
2. Select the appropriate fluorophores from the *Fluorophore* list in the *Pipetting panel* by clicking them. The selected fluorophores are listed in the *Pipette Content* field.



Note that the *Fluorophore* list contains only those fluorophores that have been selected in *Protocol Properties* in the **Protocol** view.

Remove fluorophores from the pipette by hovering over a fluorophore and clicking the X icon to return it to the *Fluorophore* list.

- Define the sample type for each fluorophore by selecting the fluorophore in the *Pipette Content* field and clicking the appropriate sample type in the *Sample Type* list. See “Sample type” on page 32.



- If necessary, add targets in the *Target* field by entering the designation and clicking the **Add** button. Within one well you can associate one target per fluorophore. Remove a target by hovering over it and clicking the **X** icon.



Note The sample types and targets in the pipette can be modified freely as you fill the layout, that is, you do not need to fully empty the pipette to modify its contents. ▲

- If required, define the replicate layout by clicking the **Replicates and Quantity Series** button and entering the number of columns and rows. See “Replicates and quantity series” on page 33.
- Click a well or paint wells on the graphical plate layout. The selected wells are filled.

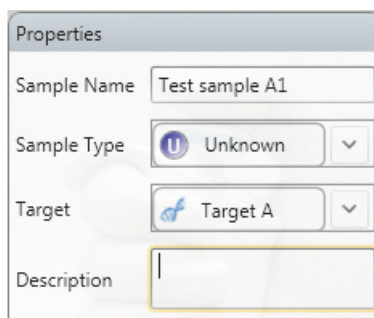
You can select a row or a column by clicking the appropriate heading and the entire plate by clicking the button in the top left-hand corner of the plate.

If you have entered a grid pattern into the *Replicates* panel, mimic that pattern when painting the target wells. You can generate several replicate sets by painting over multiples of the replicate grid area.

Once you have filled the desired wells, you can define sample properties:

- Click the **Select** button in the *Mode* action panel to change to the *Select* mode.

- Select the appropriate wells in the layout and enter their properties in the *Editing panel*.



You can enter the sample name, change the sample type and choose the target. With standards, you can enter the quantity and with AD standards, you can enter the standard value.

- Add any relevant descriptive sample information in the *Description* field.

Repeat these steps to enter the layout data. You can move freely between the *Pipette* and *Select* modes even one well at a time, if required. Fluorophores must be pipetted into the layout before the corresponding sample properties can be defined.

Sample type

The sample types are designated by a color code and an abbreviation in a circle. The following sample types are available:

Table 5–6. Sample types

Sample type	Icon	Description
Reference gene		Endogenous reference gene or housekeeping gene
Unknown		Unknown sample
Standard		Sample with known concentrations
NTC		No Template Control. A control sample.
Positive control		Positive amplification control
Negative control		Negative target control
AD/HRM Standard		Allelic discrimination or HRM standard
Empty		Empty well

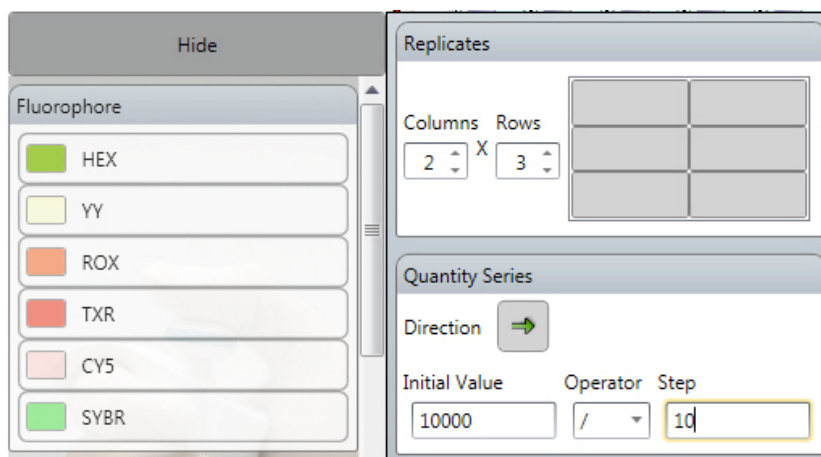
The sample type icon is displayed on top of the fluorophore bar in the layout wells.

If you selected *AD/HRM Standard* as the sample type, enter the *Standard value* field in the *Select* mode.

If you selected *Standard* as the sample type, clicking the **Replicates and Quantity Series** button presents the *Quantity Series* panel.

Replicates and quantity series

You can define replicates by entering the number of columns and rows in the *Replicates* panel. The layout pattern can be seen on the right.



You can use the *Quantity Series* panel to create a dilution series. Select the filling direction, the initial quantity, the mathematical operator for creating the series and the factor (**Step**).

In addition to using the *Replicates* panel to pipette replicates, you can mark replicates in the *Select* mode.

To mark replicates:

1. Click **Select** in the **Plate Layout** view.
2. Select the appropriate wells in the layout.
3. Click the **Define as Replicates** button in the *Tools* action panel, or right-click on the selection and select **Define as Replicates**. The replicates are marked and numbered in the layout.



Note Individual replicate sample graphs cannot be viewed separately. ▲

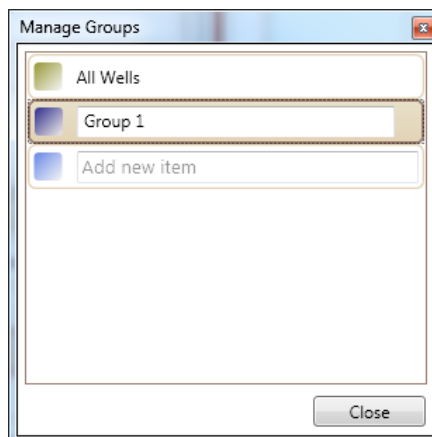
Grouping samples

You can combine several wells into sample groups if necessary. Each sample group has individual analysis options. Individual sample groups are added to the *Analysis tree* as separate items.

The created sample groups are analyzed independently of each other even when sample groups overlap.

To group samples:

1. Click **Manage Groups** in the *Tools* action panel of the **Plate Layout** view.



2. Enter the group name into the free text field and click **Add**.
3. Click **Ok** after you have entered the group names.
4. Select the appropriate wells in the plate layout.
5. Click the **Assign To Group** button in the *Tools* field and select the group name from the list, or right-click on the selection and select **Assign To Group** from the menu.

You can remove wells from a group by selecting the wells and clicking the X icon that appears on the sample group bar in the *Editing panel*.

You can edit sample group names and delete sample groups in the *Manage Groups* dialog.

Importing and exporting layouts

You can import and export plate layouts as separate files with the *.pfs* file extension. This allows you to handle layouts separately from experiments. Note that you cannot import layouts directly from an experiment.

To import a plate layout, click the **Import** button in the *Plate Layout* action panel of the **Plate Layout** view. Browse to the file and click **Open**.

To export a plate layout, click the **Export** button in the *Plate Layout* action panel of the **Plate Layout** view. Browse to the desired file location, name the file and click **Save**.

Chapter 6

Executing an Experiment

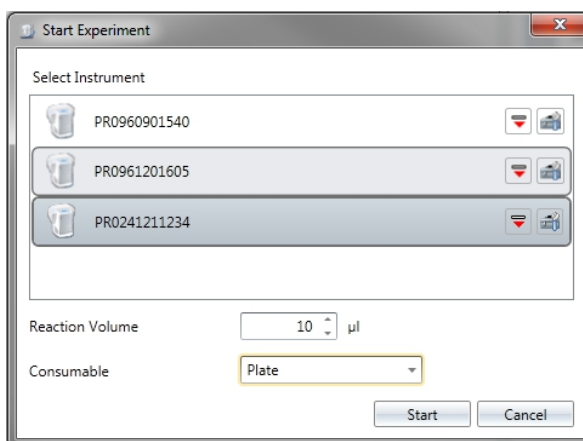
An experiment can be executed as long as you have a defined protocol with the appropriate steps and at least one available PikoReal instrument. A protocol can be run with an empty plate layout.

PikoReal Software experiment files contain the protocol, plate layout (if defined) as well as other instrument data.

The following procedure assumes that you have a PikoReal instrument already powered up and available on the network.

To execute an experiment:

1. Open the PikoReal instrument drawer and insert the reaction vessels.
2. Close the drawer and click the **Run/Rerun** button in the **Home** or **Protocol** view to initialize protocol execution.
3. If you have not saved the experiment, enter a name for the data file (*.ord*) to be created and click **Save**.
4. Select the appropriate instrument from the list in the *Start Experiment* dialog. Note that only instruments that are compatible with the sample layout are listed.



5. Specify the *Reaction Volume*.
6. If you are using a 24-well instrument, select the consumable:
 - Select **PLATE**, if you are using a 24-well plate.
 - Select **TUBES 8 to 15**, if you are using 8–15 tubes.

- Select **TUBES 16 to 24**, if you are using 16 tubes or more.



Note If you are using single tubes, you must use at least eight tubes that are placed symmetrically with at least one tube in each corner of the block. You can use empty tubes to fill the 8-tube requirement when analyzing fewer samples. ▲

7. Click the **Start** button to start protocol execution.

When protocol execution is started, the **Current Status** view opens.

Executing an existing experiment

You can rerun a previously generated experiment file (*.ord*), for example, with a different plate layout. Unless modified, the experiment rerun will contain the same analysis options as the original run.

Load the experiment by clicking the **Open** button in the **Home** view or by selecting **Open** in the application menu.

You can edit the plate layout data in the opened experiment or import a previously exported plate layout. You cannot, however, alter the protocol or change the plate type in an executed experiment. To alter the protocol, create a new experiment.

After editing, you can execute the experiment.

For the experiment to be executable, an instrument that is compatible in well format and is calibrated for the used fluorophores is required.

USB protocol transfer

Protocol files can be transferred to the PikoReal instrument using a USB memory device. After you have created a protocol, you can import the protocol file (*.ptp*) directly to a USB memory device or you can copy the protocol file to the memory device with Windows Explorer.



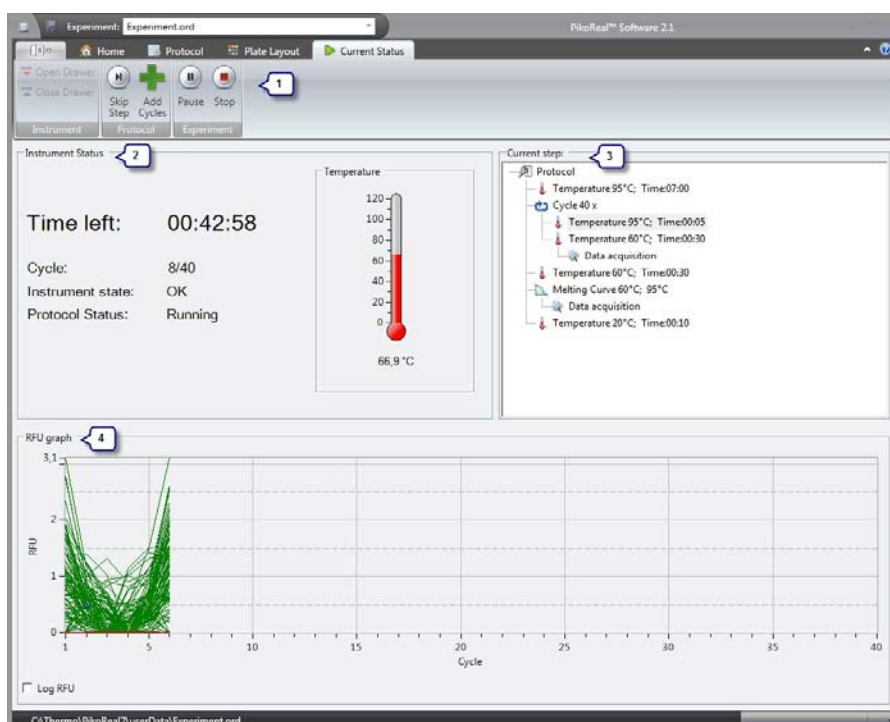
Note The name of the protocol file can contain up to eight characters, when importing it to the instrument. Protocols cannot be copied from a USB memory device to the instrument memory. Protocols executed directly from the USB memory device are not stored in the *Recent* folder in the instrument memory. ▲

Refer to the *Thermo Scientific PikoReal Real-Time PCR System User Manual* for information on running protocols from a USB memory device.

Chapter 7

Monitoring a Run

The execution of a experiment can be monitored in the **Current Status** view. This view is active only when an experiment is being executed.



If several concurrent experiments are being executed on different instruments, you can switch between them by selecting the appropriate experiment from the *Active Experiments* list in the **Home** view or from the **Experiment** menu at the top of the application window. Only one run can be viewed at a time.

The **Current Status** view contains the following main fields:

- *Action panel* (1)
Action buttons for controlling the instrument drawer, skipping protocol steps, adding protocol steps, and pausing and stopping the experiment.
- *Instrument Status* (2)
Shows the remaining run time, current cycle number, and a visual and numerical indication of the instrument temperature.
- *Current step* (3)
Highlights the current step in the protocol sequence.

- *RFU graph* (4)
Shows the sample's fluorescence intensity and updates it for each cycle in the protocol.

Adding reagents during a run

You can add reagents to a protocol under execution. The PikoReal instrument will maintain the block temperature specified in the current step when the protocol is paused for adding reagents.

To add reagents:

1. Click the **Pause** button on the action panel to temporarily halt protocol execution.
You can accomplish this also directly from the instrument's control panel by selecting the **PAUSE** command.
2. Open the instrument drawer and add the reagent.
3. Close the instrument drawer.
4. Click the **Continue** button on the action panel to resume protocol execution.
You can also select **RESUME** directly from the instrument.

Adding cycles

You can add cycles to a protocol under execution.

To add cycles:

1. Click the **Add Cycles** button on the toolbar.
2. Enter the number of cycles that you wish to add to the cycle step. The maximum number of cycles is 999.
3. Click the **OK** button to confirm the operation.



Note A warning message is displayed by the instrument, if the cycles are added during protocol execution. This information is also recorded in the log file. ▲

Protocol status

The status of a running protocol is shown on the instrument display and in the **Current Status** view.

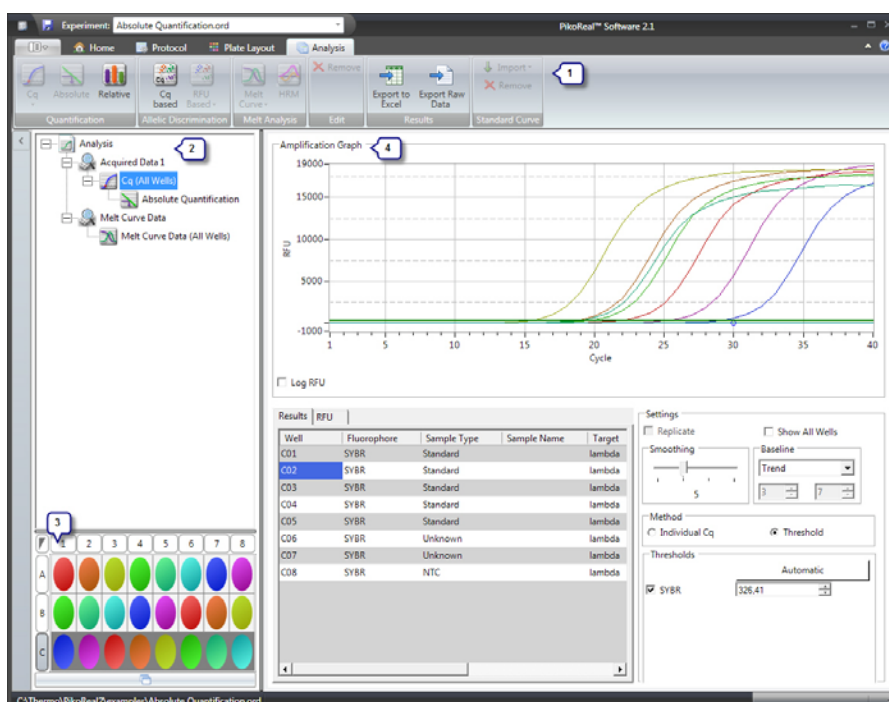
Table 7–7. Protocol status

Status	Description
Running	The protocol is under execution.
Paused	Protocol execution is paused. The instrument displays "RESUME". A running protocol can be paused from PikoReal Software by clicking the Pause button or directly from the instrument by selecting "PAUSE". While the protocol is paused, the instrument drawer can be opened and the instrument maintains the defined block temperature.
Completed	Protocol execution has completed. If the final protocol step is defined as "Infinite Hold", the block temperature is maintained at the defined level.
Stopped	The protocol is inactive. The instrument displays "WARNING". A running protocol can be stopped from PikoReal Software by clicking the Stop button or directly from the instrument by selecting "STOP".

Chapter 8

Analysis

The **Analysis** view is available for experiments that have been run. The **Analysis** view is used to perform various analyses on the fluorescence data.

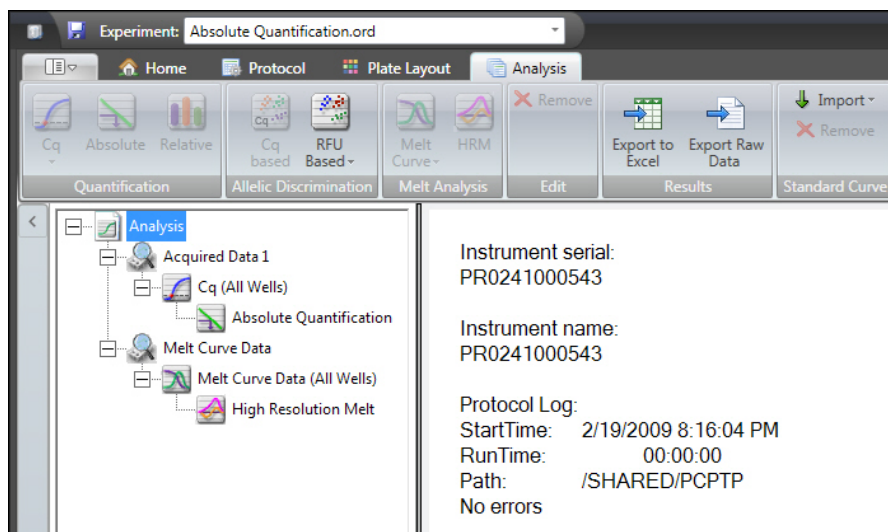


The **Analysis** view contains the following main fields:

- *Action panel* (1)
Analysis view action buttons for adding and removing analyses and exporting data.
- *Analysis tree* (2)
A hierarchical description of the selected analyses.
- *Detachable well selector* (3)
Well selector for selecting individual wells, well groups or the entire plate for result viewing. The well selector does not affect the analyses. Click the button below the selector to detach it into a separate window.
- *Analysis data* (4)
The data and settings of the selected analysis.

Run log

When first opened, the **Analysis** view shows the run log for the experiment. This log view presents instrument details and any errors that have occurred during protocol execution.



You can access this view by selecting the *Analysis* item in the *Analysis tree*.

Adding analyses

Depending on the protocol, you can add some or all of the following analyses:

- Absolute quantification
- Relative quantification
- Allelic discrimination
- Melt curve analysis
- High-resolution melt analysis

The analysis tree contains one *Acquired Data* item for each data acquisition in the protocol and a *Melt Curve Data* item, if the protocol includes a melting step. The *Acquisition Data* items are numbered consecutively.

By default, each *Acquired Data* item includes a *Cq (All Wells)* (cycle of quantification) item and a *Melt Curve Data* item includes a *Melt Curve Data (All Wells)* item. New analyses are added below a *Cq* or *Melt Curve Data* item in each data acquisition.

To add a new analysis:

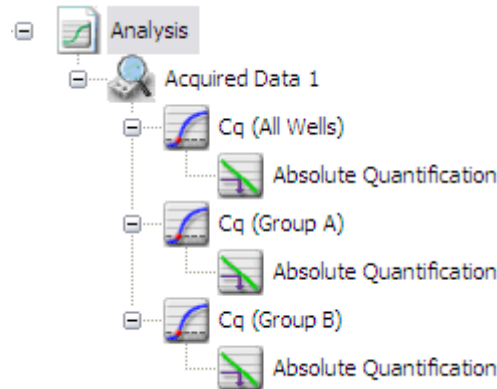
1. Select the appropriate *Cq* (or *Melt Curve Data*) item in the analysis tree.
2. Click the desired analysis button in the action panel, or right-click the selected item and select **Add** followed by the desired analysis. Only the currently selectable analyses are available.

Select the added analysis item to view its data and settings.

Note that some analysis items share settings, for example, absolute quantification shares all of its settings with its parent *Cq* item.

Analyzing sample groups

If you wish to limit an analysis to a group of samples, you need to create a new *Cq* or *Melt Curve Data* item with a sample group under which you can add the desired analyses. Sample groups are defined in the **Plate Layout** view, see “Grouping samples” on page 33.



Exporting analyses

PikoReal Software allows you to export analysis data either as a Microsoft Excel report (.xlsx) or as raw RFU data in a text file (.txt).

Note that the exported data is from the sample group currently selected in the *Analysis tree*.

To export data to Microsoft Excel, click the **Export to Excel** button in the *Results* action panel of the **Analysis** view. Browse to the desired file location, name the file and click the **Save** button.

The generated file is a comprehensive report that includes sample layout and protocol information as well as result analyses on separate worksheets. The result graphs are also included in the report.

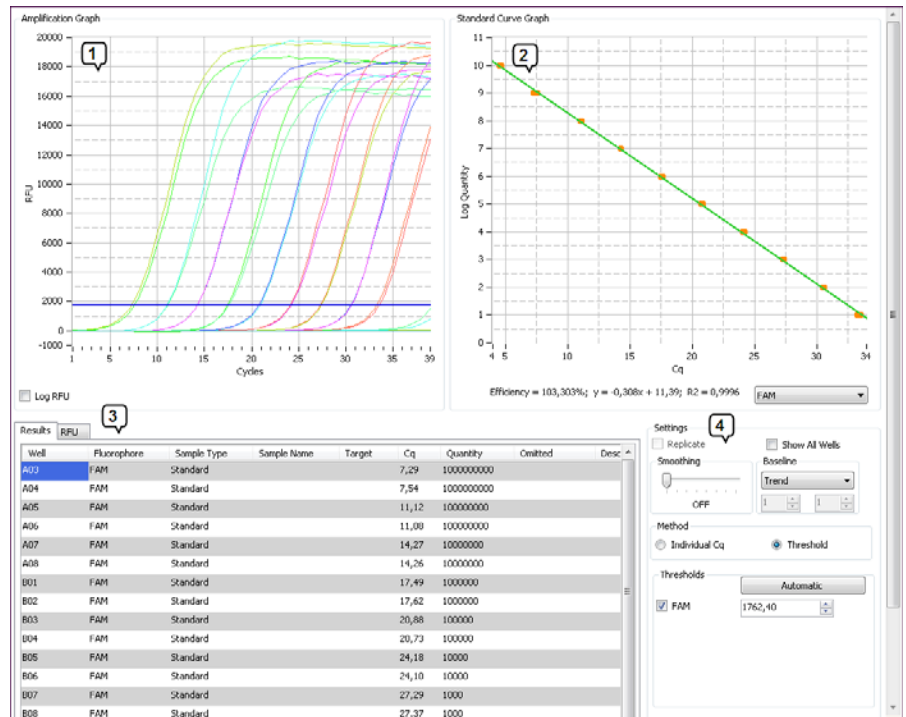
To export raw RFU data, click the **Export Raw Data** button in the *Results* action panel of the **Analysis** view. Browse to the desired file location, name the file and click the **Save** button.

Chapter 9

Cq and Absolute Quantification

The Cycle of Quantification (Cq) and Absolute Quantification analysis views present the measured fluorescence values, analysis settings, as well as the RFU (Relative Fluorescence Unit) values and results as a table.

In addition, the **Absolute Quantification** analysis view contains the *Standard Curve Graph* (shown below).



The **Absolute Quantification** analysis includes:

- *Amplification Graph* (1)
The fluorescence graphs of the samples for the selected fluorophores.
- *Standard Curve Graph* (2)
The standard curve, its equation and R^2 value, as well as the calculated reaction efficiency for the selected fluorophore. Only one fluorophore can be selected at a time.
- *Results* (3)
The absolute quantification results and RFU values for the selected fluorophores as a table.

- *Settings* (4)
 - For selecting the fluorophores for which the data is shown in the *Amplification Graph* and result fields.
 - For viewing results for replicates or all wells.
 - For applying curve smoothing.
 - For selecting the baselining method.
 - For selecting the Cq method and automatic or manual threshold settings.

You can use the *Well Selector* to select the well data to be shown. The *Settings* field provides options for presenting data for replicates or all wells.

Adding an absolute quantification analysis

To add an absolute quantification analysis:

1. In the **Analysis** view, select the *Cq* item for which the analysis is to be performed. The *Cq* item can encompass all wells or be the Cq of a distinct sample group.
2. Click the **Absolute** button in the *Quantification* action panel or right-click the selected item and select **Add** followed by **Absolute Quantification**.

The absolute quantification analysis is presented as an *Absolute Quantification* item in the *Analysis tree*. Select the analysis item to view its data and settings.

Graphs

The *Amplification Graph* presents the fluorescence graphs of the selected samples (for the selected fluorophores). The RFU intensity is presented on the Y axis and the cycles are presented on the X axis.

The *Standard Curve Graph* plots the standard sample quantities against the determined Cq values for the samples specified as standards in the plate layout. By default, the logarithmic quantities are presented on the Y axis and the Cq values on the X axis. The order of the axes can be changed by right-clicking the graph and selecting **Flip X and Y axis** from the context menu.

The *Standard Curve Graph* also shows the standard curve equation, its *R2* value and the calculated reaction efficiency.



Note When using multiple channels (multiplexing), you can have a separate standard curve for each fluorophore. ▲

Graph views

Access the different viewing options for the graphs by right-clicking above the graph view. This opens a context menu with the following options:

- *Auto Scale*
Scales the graph axes to correspond to the curve values so that the curves are shown completely. This setting can be toggled on or off.
- *Default View*
Returns a graph to the default view settings.
- *Detach graph*
Detaches the graph into a separate window.
- *Zoom In/Zoom Out*
Zooms into or out of the graph in increments of 2x. You can also zoom in by pressing the **SHIFT** key and clicking the graph, and you can zoom out by pressing the **SHIFT** key and right-clicking the graph.



Note Move the zoomed graph by pressing the **CTRL** key and clicking and dragging the graph. ▲

- *Copy*
Copies the graph as an image to the Windows clipboard, from where it can be pasted into another application.
- *Flip X and Y axis (Standard Curve Graph)*
Swaps the X and Y axes in *Standard Curve Graphs*.
- *Remove Standard/Restore Standard (Standard Curve Graph)*
Removes or restores a standard in the *Standard Curve Graph*.
- *Log RFU (Amplification Graph)*
Tick the **Log RFU** box below the RFU axis to use a logarithmic scale in the *Amplification Graph*. This setting can be toggled on or off.

Settings

You can control the settings for Cq determination and absolute quantification in the *Settings* field.

Settings

Replicate Show All Wells

Smoothing: 3

Baseline: Curve Minimum

Method: Individual Cq Threshold

Thresholds: Automatic

FAM: 1562,19

HEX: 2528,07

Tick **Replicate** to view the results based on replicates.

Tick **Show All Wells** to force the *Results* table to display all available results regardless of well selection.

Adjust the *Smoothing* slider to remove unwanted signal noise from the plotted graphs. Move the slider to the right to increase the smoothing effect. Move the slider completely to the left to disable smoothing.

Baseline

Baselining is required to compare amplification curves with different signal levels and to determine suitable threshold values. Baselining is the basis for determining Cq.

Settings

Replicate Show All Wells

Smoothing: 3

Baseline: Curve Minimum

Method: Individual Cq Threshold

Thresholds: Automatic

FAM: 1562,19

HEX: 2528,07

The **Baseline** setting has the following method options:

- *None*
No baseline is set for the curves.
This option can be used to look at the raw data and, for example, to find and exclude abnormally behaving samples, as

well as to compare reagent, primer and probe batches. This option is not available when using the *Individual Cq* method.

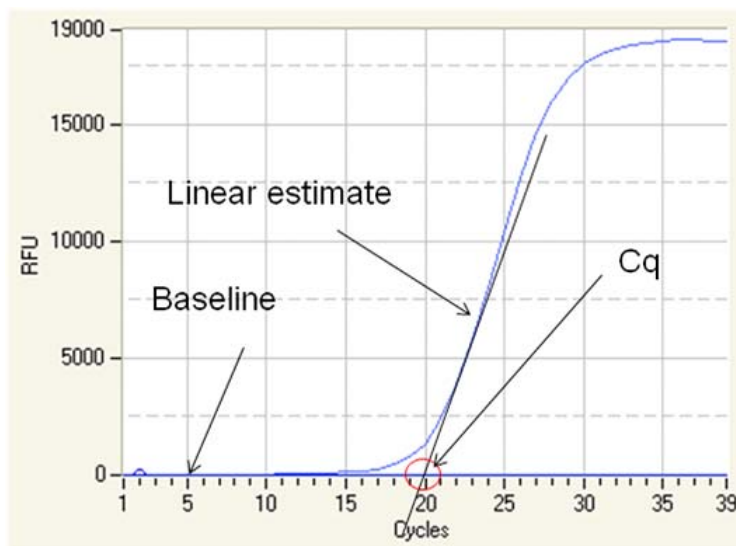
- *Trend*
The baseline of each curve is calculated based on a trend prediction algorithm. Individual curve shapes are used for baseline selection. The baseline (and whole curve) is shifted to a horizontal line and the average of the baseline value is set to zero.
- *Curve Minimum*
Individual curve minimum values are set to zero by deducting the lowest value of the curve from all other curve point values.
- *Cycle Range*
The cycles used for baseline selection are set manually. The average of the cycle range values is calculated and deducted from all curve point values.

Cq methods

PikoReal Software provides two methods for calculating the cycle of quantification: *Individual Cq* and *Threshold*. The two methods are discussed in detail below.

Individual Cq

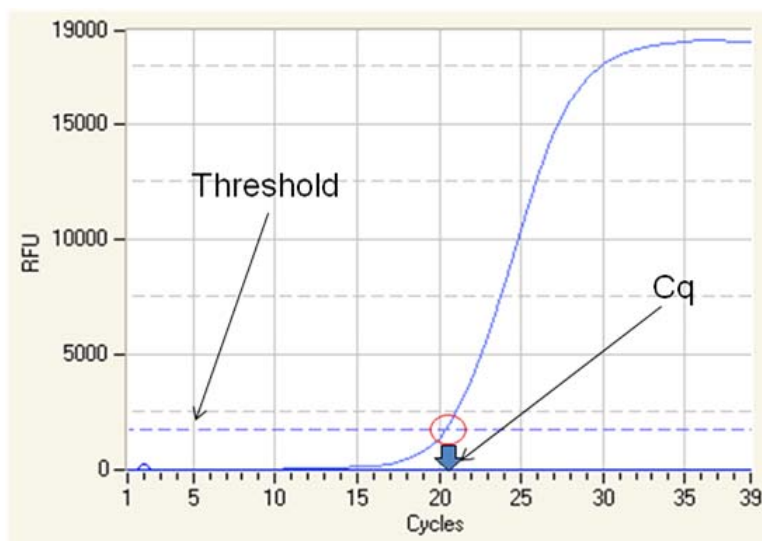
The *Individual Cq* method of calculating the cycle of quantification determines the Cq value individually for each selected fluorophore and well.



The calculation is based on the morphology of the individual curve, rather than signal strength. A linear estimate is set on the log-linear phase of the curve and the intercept point of this line and the baseline determines the Cq value.

The individual Cq method is beneficial especially when quantifying curves with significantly variable signal strengths at the same time.

Threshold In the *Threshold* method for calculating the cycle of quantification, a threshold value is set for the fluorescence values.



The threshold is presented as a line in the graph and the intercept point of the amplification graph and the threshold line determines the Cq value. The threshold lines of different channels are presented in different colors. The threshold value can be set automatically or manually.

Clicking the **Automatic** button uses an algorithm to calculate the default threshold value based on the baseline and signal noise.

You can also adjust the threshold manually by clicking and dragging the threshold line or by entering a numeric value in the threshold field of a fluorophore.



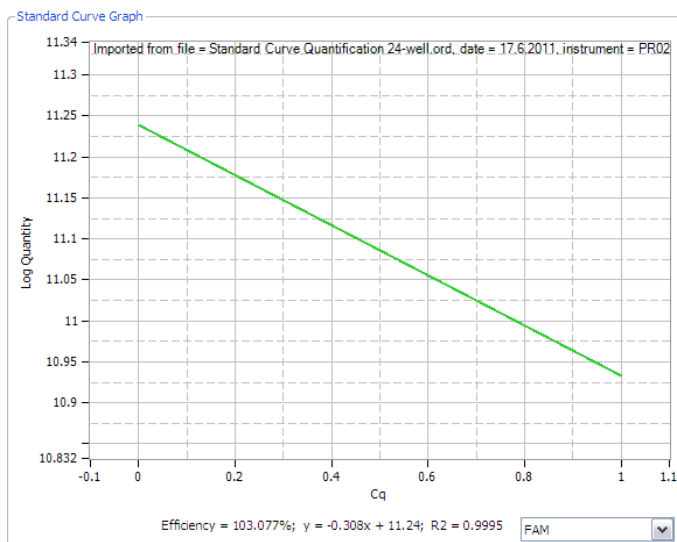
Note Whenever you change the baseline method, click the **Automatic** button to recalculate the default threshold values. ▲

Importing a standard curve

A standard curve that is created in one experiment can be used in other experiments by using the *Import* function. This feature can be used, for example, in experiments with same reaction setup, reagent batches and protocol, and in which the results are analyzed using identical analysis settings.

To import a standard curve from one experiment (source file) to another experiment (target file):

1. Open both source and target experiment files in **Home** view.
2. Activate the target file in the *Active Experiments* list.
3. In the **Analysis** view, click the **Import** button and select the source file.
4. A standard curve graph with text: “Imported from file...” appears on the target experiment’s *Standard Curve Graph*.



Note In order to verify the amplification efficiency in the target file experiment to match the amplification efficiency of the imported standard curve, a minimum of three standard samples should be included in the target file experiment. Use same analysis settings as in the source file experiment and check for the similarity of the Cq values. ▲

Results

The *Results* tab shows the results of the data analysis:

- The *Well*, *Fluorophore*, *Sample Type*, *Sample Name*, *Target* and *Description* columns show the selected well positions and the sample data entered in the layout.
- The *Cq* column shows the determined cycle of quantification. The quantity of each selected sample is calculated automatically based on its Cq and the standard curve.



Note The standard curve is created separately for each fluorophore and optionally for each sample group. ▲

- The *Quantity* column shows the specified quantities of the standards and the calculated quantities for the other samples. Based on the standard curve, the system automatically calculates the quantity for each sample not specified as a standard.
- The *Omitted* column shows the standard samples removed from the *Standard Curve Graph*.

The *RFU* tab presents the raw fluorescence data measured for each fluorophore. The data also indicates the cycle number and the temperature for each data acquisition step.

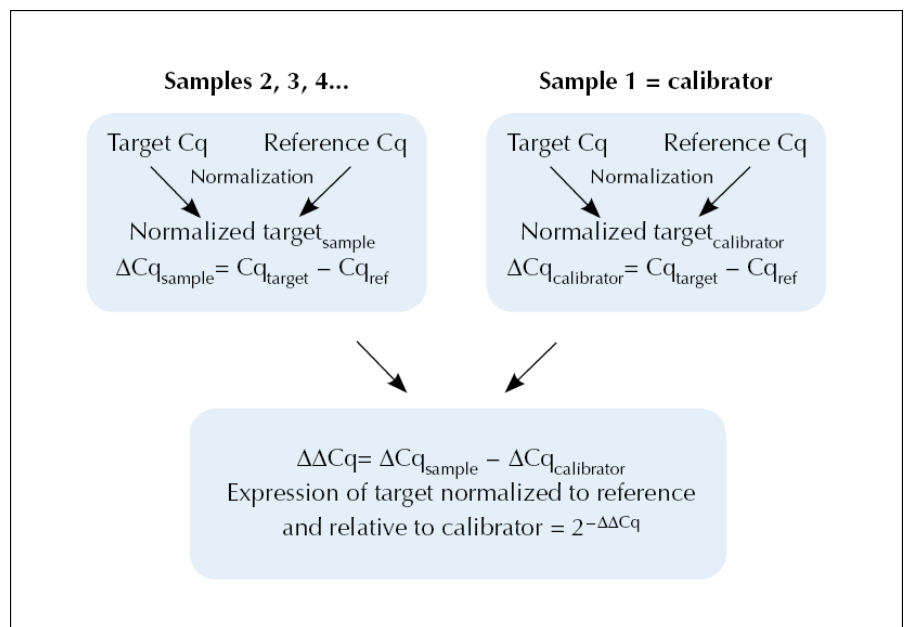
You can copy the data from the *Results* or *RFU* tab for use in other software tools by painting the desired section of the table, right-clicking and selecting **Copy** from the context menu.

Chapter 10

Relative Quantification

Relative Quantification is based on determining the ratio between the expression levels of a target molecule and one or more reference molecules in a sample, and to further compare these ratios between the sample and the calibrator. The results of the relative quantification analysis are expressed as relative quantity between the samples.

In PikoReal Software, the ratios are calculated using the $\Delta\Delta Cq$ method, which uses the Cq values.



First, the following is calculated for each sample:

$$Cq_{\text{target}} - Cq_{\text{ref}} = \Delta Cq$$

where *target* is the target molecule and *ref* is the reference molecule.

The subtraction of the Cq_{ref} values normalizes for the variation in the sample material.

If multiple reference genes are used, Cq_{ref} is calculated as a geometrical average of all reference genes: $[Cq_{\text{ref}} = (Cq_{\text{ref}1} \times Cq_{\text{ref}2} \times Cq_{\text{ref}3} \times \dots \times n)^{1/n}]$, where n = number of reference genes.

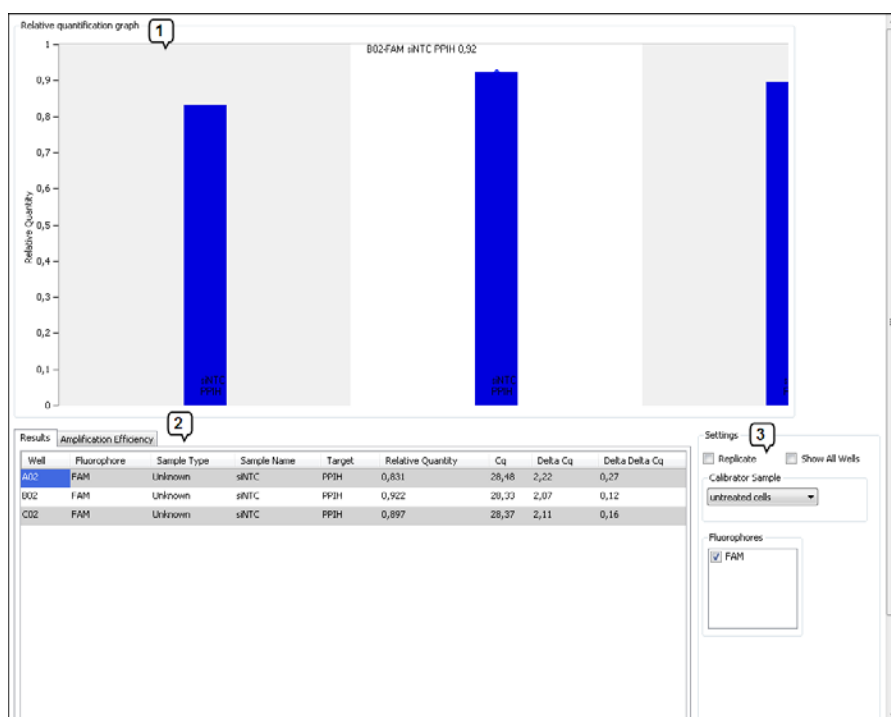
Second, one sample is designated as the calibrator sample. This is usually the sample with the lowest expression (highest Cq) or a control sample.

The ΔCq for the calibrator is then subtracted from the ΔCq of each of the other samples using equation:

$$\Delta Cq_{\text{sample}} - \Delta Cq_{\text{calibrator}} = \Delta\Delta Cq$$

The final calculation converts the $\Delta\Delta Cq$ values to a factor representing the relative quantity, that is, the difference in expression of the target molecule in the sample compared to the expression in the calibrator sample:

$$2^{-\Delta\Delta Cq} = \text{relative quantity}$$



The **Relative Quantification** analysis view includes the following main fields:

- Relative Quantification Graph (1)*
 The graph presents the results as relative quantities for each sample well or replicate group.
 Clicking the result bar for each sample well or replicate group shows the well position/replicate number, fluorophore, sample name, target and result at the top of the graph.
 For replicate groups, the sample name and target/reference is shown also on the result bars.
 In multiplex assays, each fluorophore is shown in a distinct color.
- Results and Amplification Efficiency (2)*
 Relative quantification results are displayed as a table for each sample well or replicate group, depending on the chosen display settings.

The amplification efficiency tab allows you to adjust the reaction efficiency used in the calculations.

- *Settings* (3)
The *Settings* field has options for showing the replicate groups and all sample wells, as well as settings for defining the calibrator sample and selecting the fluorophores to view.

Adding a relative quantification analysis

To add a relative quantification analysis:

1. In the **Plate Layout** view, make sure that the following information is entered in the layout data, or enter it at this stage:
 - a. Designate the reference genes with the *Reference Gene* sample type.
 - b. Identify the different genes in the layout using different *Targets*.
 - c. Designate the samples using distinct *Sample Names*.
2. In the **Analysis** view, select the *Cq* item for which the analysis is to be performed. The *Cq* item can encompass all wells or be the *Cq* of a distinct sample group.
3. Click the **Relative** button in the *Quantification* action panel or right-click the selected item and select **Add** followed by **Relative Quantification**.

The relative quantification analysis is presented by a *Relative Quantification* item in the *Analysis tree*. Select the analysis item to view its data and settings.

4. Select the calibrator sample from the **Calibrator Sample** menu in the *Settings* field. This menu lists the samples that have been given a name in the *Sample Name* field in the layout.



Note Before analyzing relative quantification data, make sure that you have valid *Cq* values. ▲

Graph views

Access the different viewing options for the graphs by right-clicking above the graph view. This opens a context menu with the following options:

- *Auto Scale*
Scales the graph axes to correspond to the curve values so that the curves are shown completely. This setting can be toggled on or off.
- *Default View*
Returns a graph to the default view settings.

- *Detach graph*
Detaches the graph into a separate window.
- *Zoom In/Zoom Out*
Zooms into or out of the graph in increments of 2x. You can also zoom in by pressing the **SHIFT** key and clicking the graph, and you can zoom out by pressing the **SHIFT** key and right-clicking the graph.



Note Move the zoomed graph by pressing the **CTRL** key and clicking and dragging the graph. ▲

- *Copy*
Copies the graph as an image to the Windows clipboard, from where it can be pasted into another application.

Amplification efficiency

The *Amplification Efficiency* tab provides the option of entering accurate values for PCR amplification efficiency to provide more accurate relative quantification results. The default value is 100%. The amplification efficiency values can be determined from a standard curve using the absolute quantification analysis and are entered per target and per fluorophore.

Settings

In the *Settings* field, you can define various view settings for relative quantification.

Tick **Replicate** to view the graph based on replicates and to view the standard deviation of replicates.

Tick **Show All Wells** to force the results table to display all available results regardless of well selection.

Select the **Fluorophores** to view by ticking them. This setting only affects the view and has no effect on the analysis.



Note The settings for determining the Cq values are described in chapter “Cq and Absolute Quantification” on page 45. ▲

Results

The following information is presented in the results table:

Well – Well designation

Fluorophore – Fluorophore name

Sample Type – Sample type as specified in the layout

Sample Name – Name given to the sample in the layout

Target – Target as specified in the layout

Description – Optional description

Relative Quantity – Relation of the starting quantities of the target and the reference material

C_q – Cycle of quantification for the sample

Delta C_q – Difference in C_q between the reference gene and the target

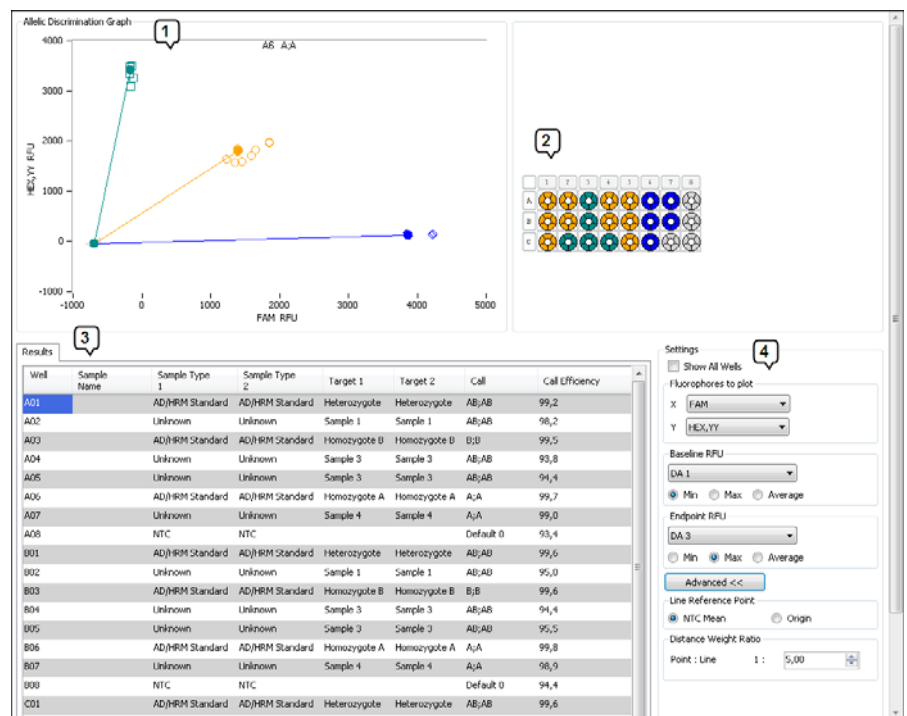
Delta Delta C_q – Difference in C_q between the calibrator and the sample

Efficiency – User-entered amplification efficiency used for the analysis

Chapter 11

Allelic Discrimination

In the **Allelic Discrimination** analysis, the RFU or C_q values of AD standard groups are compared, and samples are classified as belonging to a certain group. PikoReal Software groups the samples by calculating the distance between each sample and the averaged standard values.



The **Allelic Discrimination** analysis includes:

- *Allelic Discrimination Graph* (1)
The graph shows a colored line for each AD/HRM standard. The colored end points of the lines represent the average of the wells designated as AD/HRM standards. The colored shapes represent the values of the samples.
- *Plate graphic* (2)
The plate graphic to the right of the graph presents the results of the allelic discrimination analysis by using the same color coding as the graph.
- *Results* (3)
The results table presents the RFU values of the selected channels as well as the discrimination call and its confidence value (*Call Efficiency*).

- *Settings (4)*
Settings for assigning fluorophores to the graph, setting the zero point, etc.

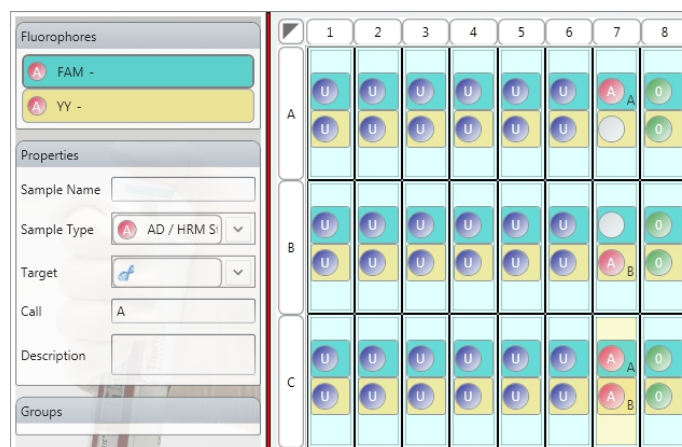
You can view the results for individual wells by selecting them in the *Well Selector*. Tick **Show All Wells**, if you wish the results table to show all available wells regardless of the well selection.

Layout creation for allelic discrimination

Notes on creating a layout for allelic discrimination analysis:

Homozygote standards must have the *AD/HRM standard* sample type with the fluorophore with which they are detected. Their call must be defined in the layout by using the *Select* mode. The other fluorophore should have the *Empty* sample type.

For the heterozygote standards, both fluorophores should be defined as *AD/HRM standards* with the corresponding homozygote calls for both fluorophores.



No template control samples must have the *NTC* sample type for both fluorophores. NTCs are recommended but not mandatory.

Adding an allelic discrimination analysis

You can base an allelic discrimination analysis on RFU or Cq values.

To add an RFU-based allelic discrimination analysis:

1. In the **Analysis** view, select the main *Analysis* item in the *Analysis tree*.
2. Click the **RFU Based** button in the *Allelic Discrimination* action panel and select the sample group or right-click the *Analysis* item and select **Add** followed by **Allelic Discrimination (RFU)** and the sample group.

The RFU-based allelic discrimination analysis is presented by an *Allelic Discrimination (All Wells)* item in the root of the *Analysis tree*. Select the analysis item to view its data and settings.

To add a Cq-based allelic discrimination analysis:

1. In the **Analysis** view, select the *Cq* item for which the analysis is to be performed. The *Cq* item can encompass all wells or be the *Cq* of a distinct sample group.
2. Click the **Cq Based** button in the *Allelic Discrimination* action panel or right-click the selected item and select **Add** followed by **Allelic Discrimination (Cq)**.

The Cq-based allelic discrimination analysis is presented by an *Allelic Discrimination* item under the chosen *Cq* item in the *Analysis tree*. Select the analysis item to view its data and settings.

Graph views

Access the different viewing options for the graphs by right-clicking above the graph view. This opens a context menu with the following options:

- *Auto Scale*
Scales the graph axes to correspond to the curve values so that the curves are shown completely. This setting can be toggled on or off.
- *Default View*
Returns a graph to the default view settings.
- *Detach graph*
Detaches the graph into a separate window.
- *Zoom In/Zoom Out*
Zooms into or out of the graph in increments of 2x. You can also zoom in by pressing the **SHIFT** key and clicking the graph, and you can zoom out by pressing the **SHIFT** key and right-clicking the graph.



Note Move the zoomed graph by pressing the **CTRL** key and clicking and dragging the graph. ▲

- *Copy*
Copies the graph as an image to the Windows clipboard, from where it can be pasted into another application.
- *Add Manual Call*
Adds a manual call that is selected from the list of defined standards.
- *Clear Manual Call*
Clears a manual call.
- *Clear All Manual Calls*
Deletes all added manual calls.
- *Virtual Standards*
Adds, removes or edits virtual standards the *Standard Edit Dialog*.

Settings

In the *Settings* field, you can define settings for the allelic discrimination analysis. Note that the settings differ based on whether you are performing an RFU- or Cq-based analysis.

In the *Fluorophores to plot* field, you can select the fluorophores to plot for the X and Y axes.

In the *Baseline RFU* and *Endpoint RFU* fields, which are available only for RFU-based analyses, you can select the starting and ending steps for data detection. By default, *Baseline RFU* is set to *Min* and *Endpoint RFU* is set to *Max*, and the software calculates the difference between the minimum and maximum signal values.

The *Line Reference Point* setting controls the starting point of the graph lines. *NTC Mean* sets the starting point of the lines at the average value of all NTC type samples. This reduces the effect of background signal alterations, which result from variations in the chemistry used in the analysis. *Origin* sets the starting point of the lines to the graph origin.

The *Distance Weight Ratio* field allows you to adjust how PikoReal Software groups the samples relative to the standards based on the distance of the sample data to the point or the line of the AD standard group.

Virtual standards

You can add standards to the analysis manually and edit the standard group.

To add a standard, right-click on the *Allelic Discrimination Graph* and select **Virtual Standards** followed by **Add Virtual Standard** from the context menu. An ellipse is drawn in the graph around the new standard point.

Edit the new virtual standard point by right-clicking on the graph and selecting **Virtual Standards** followed by **Edit Virtual Standard**. This opens the *Standard Edit Dialog*.

Enter a name for the new virtual standard in the *Identifier* field. You can alter the shape of the standard also by clicking and dragging the ellipse.

Move the standard to the desired position in the graph by clicking and dragging the standard point.

You can delete a virtual standard by right-clicking on the graph and selecting **Virtual Standards** followed by **Remove Virtual Standard**, and selecting the virtual standard that you wish to delete.

Manual call

Add a manual call to a sample by right-clicking and selecting **Add Manual Call** and choosing the standard. Remove individual manual calls by selecting **Clear Manual Call** in the context menu. Select **Clear All Manual Calls**, if you wish to delete every added manual call.

Chapter 12

Melt Curve

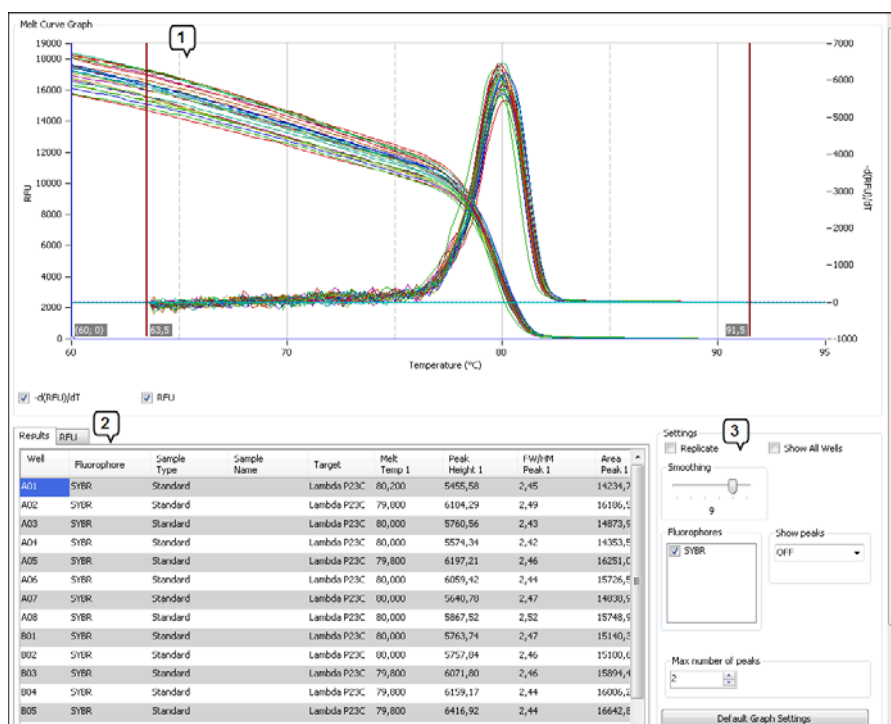
The melt curve is a post-PCR analysis step performed on an amplified product. Typically, the analysis follows the amplification step and it utilizes an interchelating/dsDNA-binding dye, such as SYBR Green I.

The amplified product is subjected to a gradual increase of temperature, from the annealing temperature to about 95°C, while monitoring the fluorescence value every 0.25–0.5°C.

As a result of the temperature increase, the amplified product dissociates, that is, melts to form single-stranded DNA strands. The dsDNA binding dye molecules are released, which leads to a decrease in fluorescence.

The melting temperature, T_m , of the product is defined as the temperature at which half of the dsDNA products have melted. The melting temperature is a characteristic value for the amplification and it is calculated from the melting curve data by plotting the first negative derivative of the fluorescence plot.

The melt curve is used as a qualitative analysis of DNA products, for example, in the identification or characterization of PCR products. In addition, the analysis allows the user to check for unwanted PCR products, such as primer dimers and secondary amplification products and thus provides a tool for assay optimization.



The Melt Curve analysis view includes the following main fields:

- *Melt Curve Graph* (1)
The graph plots fluorescence values relative to the temperature and/or the first negative derivative of the fluorescence plot.
- *Results* (2)
The results are presented as a table with melt temperature, peak height, peak area and full-width/half-magnitude ratio information for the selected fluorophores.
- *Settings* (3)
 - For selecting the fluorophores for which the data is shown.
 - For viewing results for replicates or all wells.
 - For applying curve smoothing.
 - For showing the located peaks on the graph.
 - For selecting the number of peaks for which the data is shown in the results table.

Adding a melt curve analysis

A melt curve analysis that encompasses all layout wells [*Melt Curve Data (All Wells)*] is automatically added to the *Analysis tree* when the protocol includes a *Melt Curve* step.

To limit the melt curve analysis to a certain sample group:

1. Select the *Melt Curve Data* item in the *Analysis tree*.
2. Click **Melt Curve** in the *Melt Analysis* action panel or right-click and select **Add** followed by **Melt Curve**.

3. Select the sample group from the list.

The melt curve analysis is presented by a *Melt Curve Data* item in the *Analysis tree*. Select the analysis item to view its data and settings.

Graph views

Access the different viewing options for the graphs by right-clicking above the graph view. This opens a context menu with the following options:

- *Auto Scale*
Scales the graph axes to correspond to the curve values so that the curves are shown completely. This setting can be toggled on or off.
 - *Default View*
Returns a graph to the default view settings.
 - *Detach graph*
Detaches the graph into a separate window.
 - *Zoom In/Zoom Out*
Zooms into or out of the graph in increments of 2x. You can also zoom in by pressing the **SHIFT** key and clicking the graph, and you can zoom out by pressing the **SHIFT** key and right-clicking the graph.
- Note** Move the zoomed graph by pressing the **CTRL** key and clicking and dragging the graph. ▲
- *Copy*
Copies the graph as an image to the Windows clipboard, from where it can be pasted into another application.



Settings

Select the curves to view by ticking the boxes below the graph:

- Tick **RFU** to display the detected fluorescence. This curve typically decreases in signal intensity as the temperature increases.
- Tick **-d(RFU)/dT** to display the negative first derivative of the RFU curve. The curve presents the change in detected fluorescence relative to the change in temperature. The peak is used to determine the melting point. The derivative curve calculation algorithm automatically corrects for signal intensity decrease that is unrelated to DNA melting.

You can select to view individual fluorophores from the *Fluorophores* field and individual wells from the *Well Selector*. Tick **Show All Wells**, if you wish the results table to show all available wells regardless of the well selections.

Adjust the *Smoothing* slider to remove unwanted signal noise from the plotted graphs. Move the slider to the right to increase the smoothing effect. Move the slider completely to the left to disable smoothing.

Peaks

You can view the located peaks on the derivative curve $[-d(RFU)/dT]$ by selecting a channel with the *Show peaks* control.

Use the *Max number of peaks* control to limit the number of detected peaks within the range of 1–4.

You can also set the temperature range by adjusting the position of the low and high temperature limit bars on the graph.

Set the peak de the graph. Use this control to reduce the effect of signal noise.

The peak detection threshold and temperature range can be reset to their default values by pressing the *Default Graph Settings* button.

Results

The results table presents the calculated melting curve data.

The following information is presented for each peak:

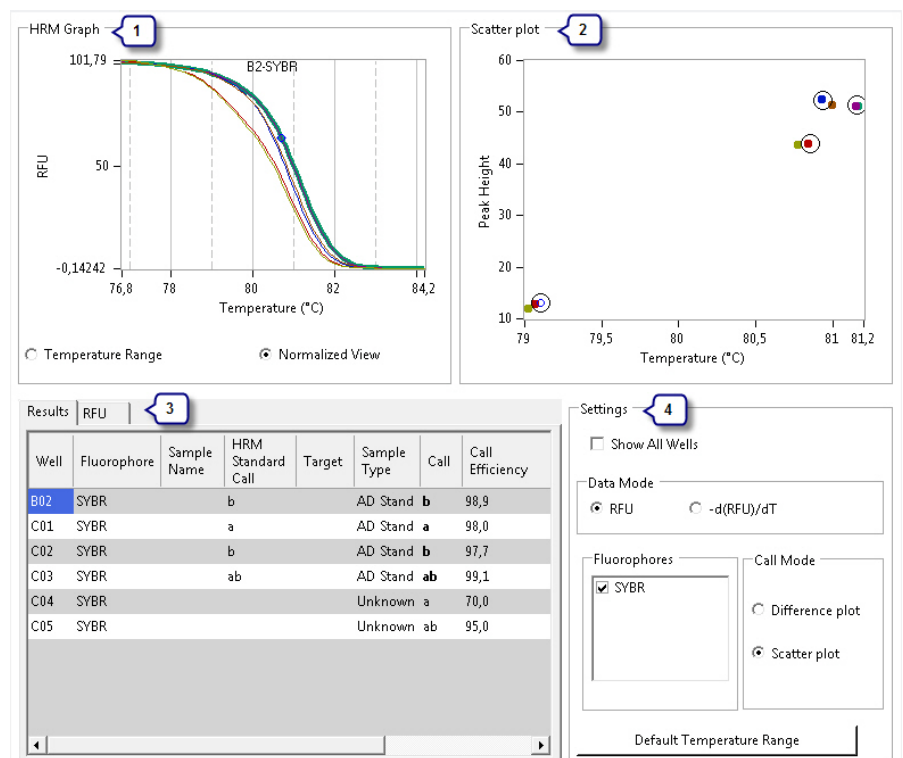
- *Melt Temp* – Detected melting point
- *Peak Height* – Highest value of the peak
- *FW/HM Peak* – Full-width/half-magnitude ratio
- *Area Peak* – Calculated peak area

Chapter 13

High-resolution Melt Analysis

HRM or high-resolution melt analysis enables melt curve shape based identification of DNA products. HRM analysis is typically used for mutation analysis due to its capability to distinguish between single nucleotide differences.

The HRM analysis view features two distinct visualization methods for normalized data. PikoReal protocols can have up to 25 data points per temperature degree which facilitates HRM analysis.



The HRM view includes the following main fields:

- HRM Graph (1)**
 The graph plots relative fluorescence against the temperature. You can view the graph with the full temperature range or as a normalized graph according to the selected temperature range.
- Scatter Plot or Difference Graph (2)**
 The scatter plot visualizes analysis data based on detected melt curve peaks and their relative heights and locations on the temperature axis.
 The difference graph visualizes analysis data as the difference

between individual melt curves and a virtual curve based on all available melt curves.

- *Results* (3)
The results are presented as a table with calculated HRM call and its efficiency.
- *Settings* (4)
 - For selecting the source data for the HRM analysis.
 - For selecting the fluorophores for which the data is shown.
 - For selecting the analysis method between the scatter plot and difference graph.



Note Melt analysis settings including peak detection and smoothing affect the HRM analysis source data. ▲

Adding an HRM analysis

An HRM analysis can be added to a *Melt Curve Data* analysis item; either to all layout wells [*Melt Curve Data (All Wells)*] or to a *Melt Curve Data* analysis that is limited to a certain sample group.

To add an HRM analysis:

1. In the **Analysis** view, select the *Melt Curve Data* analysis item for which the analysis is to be performed.
2. Click the **HRM** button in the *Melt Analysis* action panel or right-click the selected item and select **Add** followed by **High Resolution Melt**.

The high-resolution melt analysis is presented by an HRM item in the *Analysis tree*. Select the analysis item to view its data and settings.

HRM analysis and results

The *HRM Graph* shows the selected source data (*Data Mode*) for the high-resolution melt analysis. The *HRM Graph* includes an algorithm that automatically corrects for signal intensity decrease that is unrelated to DNA melting.

HRM analysis requires that standards are defined with the *AD/HRM standard* sample type in the **Plate Layout** view, and that a distinct call is given for each different standard. PikoReal Software automatically calls all unknown samples to the standard with which they have the highest correlation.

Use the vertical temperature range bars in the *HRM Graph* to limit the area of interest, that is, the area of DNA melting. The area within the lower and upper range bars is used to normalize the data on the relative fluorescence axis. PikoReal Software automatically defines a default range based on the data.

After setting the analysis limits, you can select *Normalized RFU/DfDt* to view the data used for the HRM analysis. This data is automatically normalized for the temperature axis.

The scatter plot and the difference graph are based on normalized data. Tick *Standards* to plot the average of the *AD/HRM Standards*.

The results table presents the sample calls based on the analysis in the *Call* column. The call efficiency is the ratio of the distance between an individual sample and the closest standard and the second closest standard presented as a percentage.

Graph views

Access the different viewing options for the graphs by right-clicking above the graph view. This opens a context menu with the following options:

- *Auto Scale*
Scales the graph axes to correspond to the curve values so that the curves are shown completely. This setting can be toggled on or off.
- *Default View*
Returns a graph to the default view settings.
- *Detach graph*
Detaches the graph into a separate window.
- *Zoom In/Zoom Out*
Zooms into or out of the graph in increments of 2x. You can also zoom in by pressing the **SHIFT** key and clicking the graph, and you can zoom out by pressing the **SHIFT** key and right-clicking the graph.



Note Move the zoomed graph by pressing the **CTRL** key and clicking and dragging the graph. ▲

- *Copy*
Copies the graph as an image to the Windows clipboard, from where it can be pasted into another application.

Settings

The *Data Mode* setting allows you to change HRM source data from the default RFU to the first negative derivative of RFU data (*-DffDt*). This alters the results slightly.

The *Fluorophores* field allows you to select one fluorophore at a time.

The *Call Mode* selector allows you to choose the analysis method between the scatter plot and difference graph.

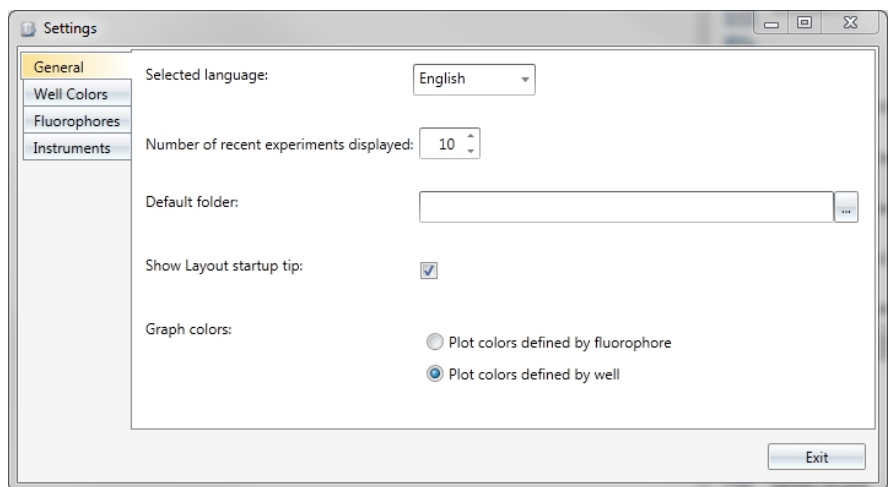


Note All melt curve analysis settings affect the input data for the HRM analysis including graph controls. ▲

Chapter 14

Settings

To access PikoReal Software settings, click the **Settings** button in the **Home** view. In the *Settings* dialog, you can configure general software settings, define well and fluorophore colors, and set instrument addresses and names.

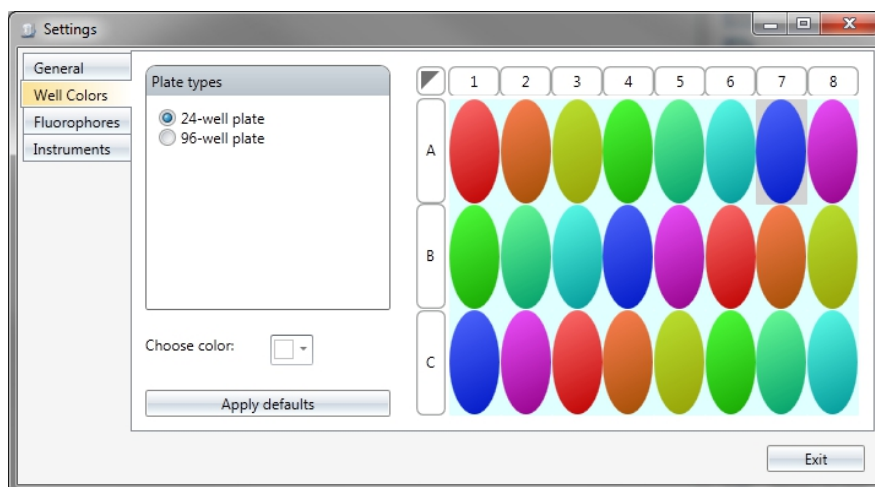


In the *General* settings, you can:

- Set the user interface language.
- Define the number of experiments displayed in the *Recent Experiments* list in the **Home** view.
- Set the default folder for PikoReal Software.
- Choose whether to display the tip box in the **Plate Layout** view.
- Select the method of defining colors used for plotting graphs.

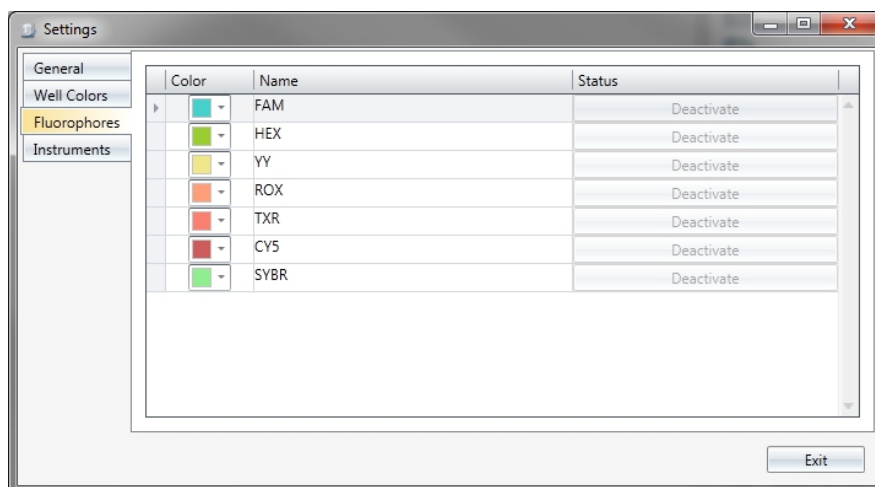
Well colors

In the *Well Colors* settings, you can define the colors used to present the wells in the layout. These also define the graph plotting colors, if set to do so in the *General* settings.



Fluorophores

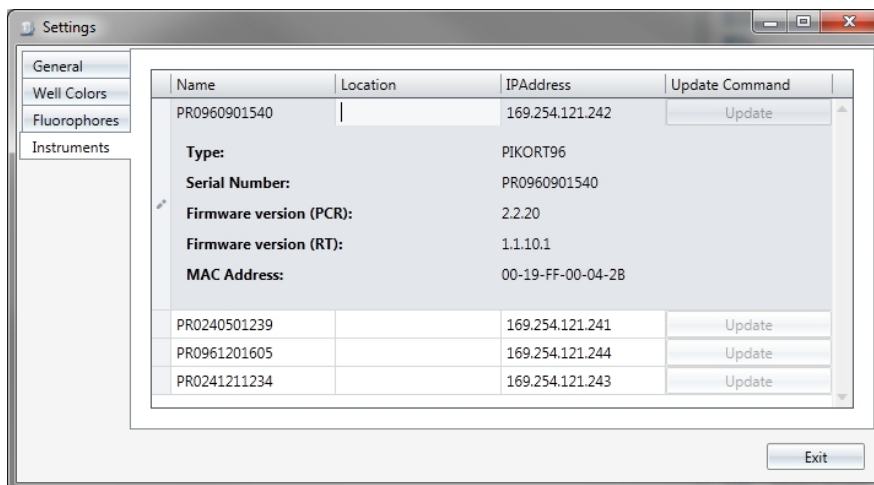
In the *Fluorophores* settings, you can set the colors used to present the different fluorophores. These colors are used in graph plotting, if defined to do so in the *General* settings.



You can remove user-entered fluorophores by clicking the *Delete* button. This removes the fluorophores from the *Protocol Properties* view and correspondingly from the list of available fluorophores when pipetting in the **Plate Layout** view.

Instruments

The *Instruments* settings lists the instruments that are currently available on the network. Each instrument has a freely editable name and location, as well as a configurable IP address.



To change the name, location or IP address of a listed instrument:

1. Select the instrument in the list.
2. Edit the properties (*Name*, *Location* and *IP Address*) in the fields.
3. Click **Update** to send the updated information to the instrument.

Note that it may take several minutes for an updated instrument to reappear in the instrument list.

Appendix A

Color Calibration

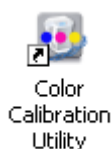
The *Color Calibration Utility* allows you to add new fluorophores to an instrument and recalibrate existing fluorophores. An instrument's default fluorophores have been precalibrated at the factory. The precalibrated fluorophores can be recalled from the instrument's memory even after recalibration, if necessary.

To perform a color calibration:

1. Launch the *Color Calibration Utility* from the **Start** menu:

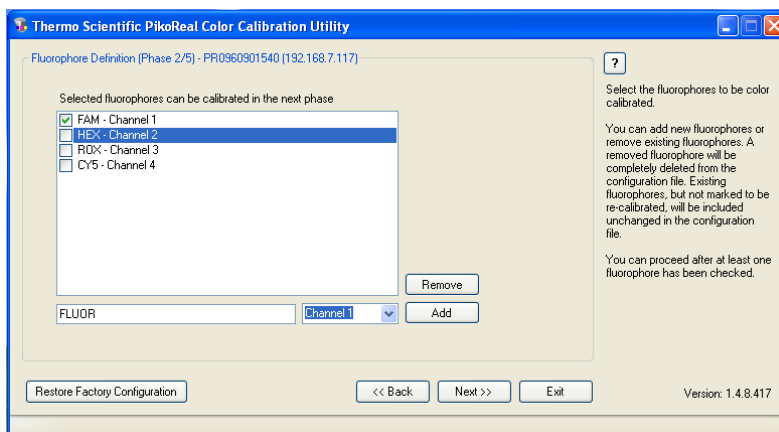
Start > All Programs > Thermo Scientific PikoReal Software 2.1 > **Color Calibration Utility**,

or by double-clicking the desktop icon.



Click the question mark button for helpful information on each phase of the calibration process.

2. Begin by selecting the instrument to be calibrated and click **Next**.
3. Tick the fluorophores for which the instrument is to be calibrated. Untick the fluorophores for which you wish to maintain the previous calibration.

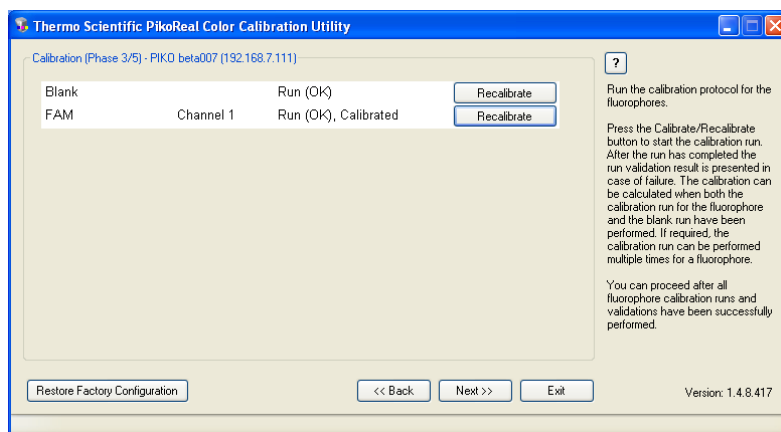


Add a new fluorophore by entering its name into the text field, choosing the channel and clicking the **Add** button.

Here you can also restore an instrument to its factory calibration state by clicking the **Restore Factory Configuration** button. Use this function with caution, as it will delete all fluorophore calibration data that has been created after the instrument has been taken into use.

Click **Next** to proceed to the next phase.

4. You are presented with the list of fluorophores for which the instrument is to be calibrated.



The first item “Blank” is always on the list, since baselining is necessary each time a fluorophore calibration is performed.

Baselining is performed by filling a plate with the buffer solution recommended by the fluorophore supplier. The recommended buffer solution volume is 10 µl.

Click the **Calibrate** button to perform the calibration for the blank run. A successful blank run is indicated as “Run (OK)”.

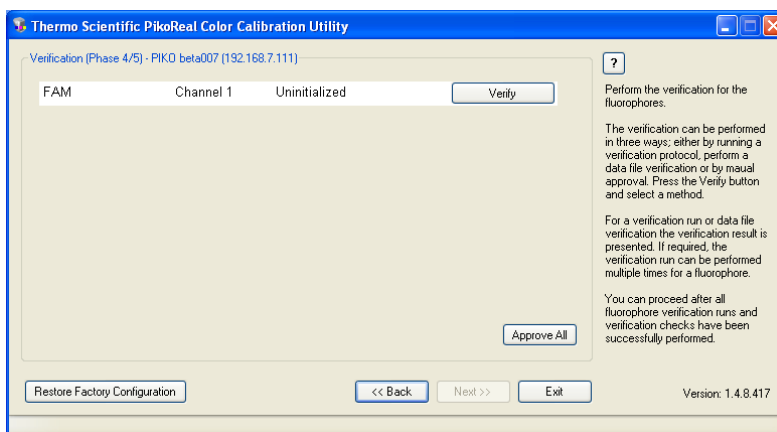
When calibrating existing fluorophores and adding new fluorophores, use the same buffer solution and prepare a concentration which provides a clear signal in the primary channel. The signal should be about 10,000 RFU. Signals of over 55,000 RFU are not accepted.

Click the **Calibrate** button to perform the calibration for each fluorophore. A successful calibration is indicated as “Run (OK), Calibrated”.

You can proceed to the next phase after the blank run and all of the selected fluorophore calibrations have been completed successfully.

Click **Next** to proceed to the verification phase.

5. The fluorophore calibrations must be verified before they are sent to the instrument. You can verify them in one of three ways:
 - Perform a verification run using actual chemistry,
 - Verify the calibration using an existing data file, or
 - Manually approve the calibration.

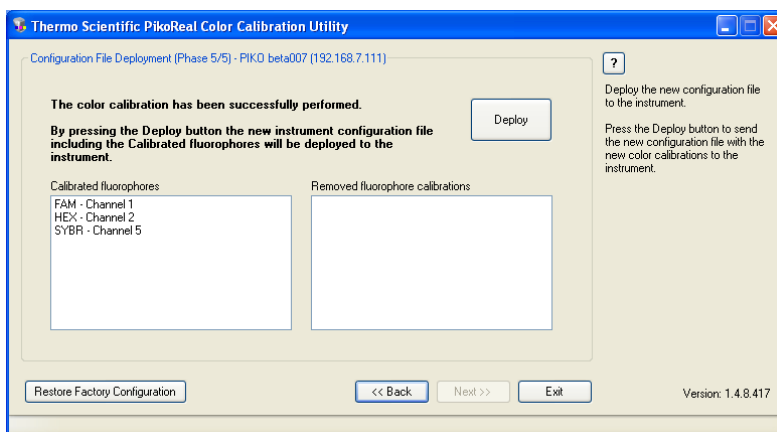


Click the **Verify** button and select the verification method.

You can also immediately verify all of the calibrations that have been performed by clicking the **Approve All** button.

Click **Next** to proceed to the final step after all of the calibrations have been verified.

6. The calibrations have now been completed and you can deploy the data to the instrument.



Click the **Deploy** button to send the new calibration data to the selected instrument.

The color calibration has now been completed and you can close the *Color Calibration Utility*.

Index

- absolute quantification, 45
- action panel, 16
- AD/HRM standard, 33, 59, 69
- adding analyses, 42
- adding cycles, 38
- adding reagents, 38
- allelic discrimination, 59
- amplification efficiency, 51, 56
- amplification graph, 45
- analyses, 42
- Analysis** view, 41
- analyzing sample groups, 43
- baseline, 48
- channels, 27
- color calibration, 75
- connecting instruments, 12
- consumable, 35
- Cq, 57
- Cq determination, 49
- creating a layout, 30
- creating a protocol, 22
- Current Status** view, 37
- cycle range baseline, 49
- Cycle step, 25
- data acquisition, 26, 27
- data file, 35
- delta Cq, 57
- delta delta Cq, 57
- detection channels, 27
- editing a protocol, 22
- Editing mode, 30
- Editing panel, 29
- empty plate layout, 35
- executing experiment, 35
- existing experiment, 36
- experiment execution, 35
- experiment protocol, 21
- exporting analyses, 43
- exporting plate layouts, 34
- exporting protocols, 28
- fluorescence graph, 45
- fluorophore, 24, 30, 72
- global minimum baseline, 49
- graphs, 46
- grouping samples, 33
- heated lid, 24
- high-resolution melt analysis, 67
- hold time, 28
- Home** view, 16, 17
- HRM, 67
- importing plate layouts, 34
- importing protocols, 28
- individual Cq, 49
- infinite hold, 26
- installing PikoReal Software, 11
- instrument IP address, 13, 73
- instrument name and location, 13, 73
- instrument status, 39
- instruments, 12, 73
- IP address, 13, 73
- launching PikoReal Software, 15
- layout creation, 30
- layout of samples, 29
- lid, 24
- manual call, 62
- melt curve, 63
- Melt Curve step, 27
- minimum hold time, 28
- monitoring a run, 37
- multiplexing, 46
- name of instrument, 13
- navigating PikoReal Software, 16
- navigation bar, 16
- network settings, 12
- ord*, 35
- PC requirements, 11
- peak, 66
- PikoReal Software, 9
- PikoReal Software installation, 11
- Pipette** button, 30
- Pipetting panel*, 29
- plate layout, 29

- plate layout creation, 30
- Plate Layout** view, 29
- plate well, 29
- protocol, 21
- protocol creation, 22
- protocol editing, 22
- protocol properties, 24
- protocol status, 39
- protocol steps, 22
- Protocol** view, 21, 37
- qPCR graph, 54
- quantity series, 33
- ramp rate, 24, 26
- reaction volume, 24
- relative quantification, 53
- relative quantity, 57
- replicate, 31, 33, 56
- RFU, 46
- run log, 42
- run monitoring, 37
- running experiment, 35
- sample group, 33, 43
- sample type, 31, 32
- settings, 71
- single tubes, 36
- smoothing, 48, 66
- software upgrade, 11
- standard, 33
- standard curve, 50
- standard curve graph, 45
- starting PikoReal Software, 15
- status of protocol/instrument, 39
- steps, 22
- Temperature step, 25
- threshold, 46, 50, 64, 68
- trend baseline, 49
- upgrading software, 11
- USB protocol, 36
- USB-to-Ethernet adapter, 12
- user interface, 15
- well, 29
- well colors, 72
- Well Selector*, 60, 65
- views, 19
- Virtual Pipetting, 29
- Virtual Pipetting mode, 30
- virtual standard, 62

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