Operator Manual

Rotor-Gene™ 3000

Real Time Thermal Cycler

corbett RESEARCH

6.0.38

Table of Contents

1 Disclaimer

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2 Introduction

2.1 Welcome

Welcome to the Rotor-Gene version 6.0.38 software. This help manual will instruct the user through run setup and analysis.

The Rotor-Gene real time DNA amplification system is an open chemistry platform allowing the use of any real time chemistry. The software therefore aims to provide support for analysis of data obtained any chemistry available. Check the What's New section of this manual for an update on changes and novel features in this version of the software.

2.2 Running the Rotor-Gene 2000

The Version 6.0.38 software does not support operation of the Rotor-Gene 2000 machine. This version can be run in virtual mode, and used for analysis of files run on these machines. Please refer to the documentation of the Version 5 operating software for information on the use of these machines.

2.3 What's New

What's New in Version 6?

This topic explains the new features in version 6 of Rotor-Gene, and answers commonly asked questions about the location of existing features that have been moved. These features incorporate design changes and requests by users.

Analysis Change: Quantitation and Comparative Quantitation

Improvements have been made to the normalisation and statistical algorithms used in both Quantitation and Comparative Quantitation. This will affect C_T values, Takeoff cycles, and calculated concentrations on existing runs. The changes use improved statistical models to more accurately calculated unknown concentrations. You should revalidate any existing procedure using these formulae before using in a production environment.

Analysis Change: M and B values rearranged to match competitor values
To facilitate the use of published materials, the Rotor-Gene software is changing its reported M and B values to match those used by the competitor standard curve arrangement, that is, "C_T = log(conc) * m + b"

As a rearrangement of equations, the former M and B values are still available from the standard curve in the "conc=10^(mx + b)" format on the Quantitation window, and in the reports.

Visually Group Replicates
Samples can now be grouped on the display by right-clicking on any graph and selecting "Group By Replicates".

Operator and Analyst Access Levels
Windows Administrators can now define access levels for users on their local computer, or on the entire network. This allows giving permission to perform runs or analysis only to certain users. Samples can be locked at the beginning of a run and then only unlocked by an Administrator, enabling you to enact strong security policies in the laboratory.

Two Standard Curves Relative Quantitation Method Added
The Two Standard Curves method is now available in the software in the new Relative Quantitation module.

Delta Delta C_T Relative Quantitation Method Added
The Delta Delta C_T method is now available in the software in the new Relative Quantitation module.

New Quick Start Wizard Added
To aid new users of the system, a quick start wizard has been added, featuring a rotor-centric sample editor. A default run filename is now generated based on the template filename, the current date and the run number for the day. This reduces the number of keyboard entry required to begin a run.

Improved Auto-Stat Statistics
The result grid statistics now calculate differently, based on the selected column. For calculated concentrations in Quantitation, the mean and standard deviation are calculated to take into account the exponential statistics model, and so give more meaningful measures of variance. These use orders of magnitude for standard deviation, and the geometric mean for the average.

Statistics calculated for replicates
Replacing the C_T standard deviation, a more comprehensive replicate framework has been put in place. Samples with identical names are now treated as replicates, and will have confidence intervals generated for estimated concentrations, C_T values and other calculated results.

Export to LinReg
It is now possible to export Quantitative data directly to LinReg. Since LinReg uses normalised quantitation data, you will need to create a quantitation analysis before exporting.

Export All Analysis To Excel
The results of all analysis can be automatically sent to Excel. Extensions exist to allow this data to be sent to LIMS systems.

User-defined sample groups
To facilitate toggling blocks of samples, a Sample Groups feature enables the user to group related samples. These can then be turned on or off by right-clicking on the sample selector. Sample groups will be eventually used to allow grouping for more complex forms of relative

quantitation.

Per-channel sample definitions
It is now possible to create completely separate sample definitions for each acquired channel, using the new sample pages feature. This allows greater flexibility in designing multiplex runs, and cleaner result tables for simplex reactions running multiple standard curves.

Concentration Measurement
This new analysis technique enables you to read DNA concentrations on the Rotor-Gene. Comprehensive statistics are generated, enabling you to have confidence in your results.

Add Support for Nested Amplification
It is now possible to set the rotor speed at each step, to either high, normal or still on the Rotor-Gene. This enables customers to perform nested amplification.

Cycling Profiles Can Be Chained
To allow for cycling profiles which vary substantially in the middle, such as those used for Nested amplification, it is now possible to connect different cycling steps together to create one set of data to be quantitated. This feature now effectively enables any conceivable cycling profile to be programmed.

Annealing Analysis
Annealing Analyses are a useful tool in detecting unexpected additional products in amplification reactions, and are added in this version of the software. This is achieved by setting the profile to ramp downwards in temperature, and then performing melt analysis with the vertical scale flipped.

User Interface Enhancements
Easier toggling of options when displaying two quantitation analyses side-by-side with the drop-down menus available for all the hidden toolbar buttons.

Improved handling of concentrations in scientific notation, and value formatting in general.

In result grids, by right-clicking on column headings, the options can be toggled on or off. Only the selected columns will appear in printed reports, allowing a degree of per-site customisation.

Sample Editor now has Undo button.

Temperature graph can display time axis in real time, not just minutes elapsed since the run start.

The analysis selection window has been made more compact.

Printed reports now paginate better.

Export To Any Application
It is now possible to export data from the Rotor-Gene application into any third party application, with the pluggable export filters mechanism. This enables the Rotor-Gene software to seamlessly integrate with integrated diagnostics systems and facilitate analysis in third-party software such as Matlab and statistical applications.

Can Import Standard Curve From Outside Sources
The Standard Curve formula can now be input directly, to allow import of standard curves averaged over many runs, for example.

Fixed "Access Denied" Error on Windows NT 4.0
Access Denied errors no longer occur when opening small REX files on Windows NT 4.0

Corrected Intermittent Overflow Error
When the operating computer was running for longer than 28 days, an Overflow error could appear on some computers intermittently. This has been fixed in this release.

2.4 Installation and Maintenance

2.4.1 Installation

Hardware Installation

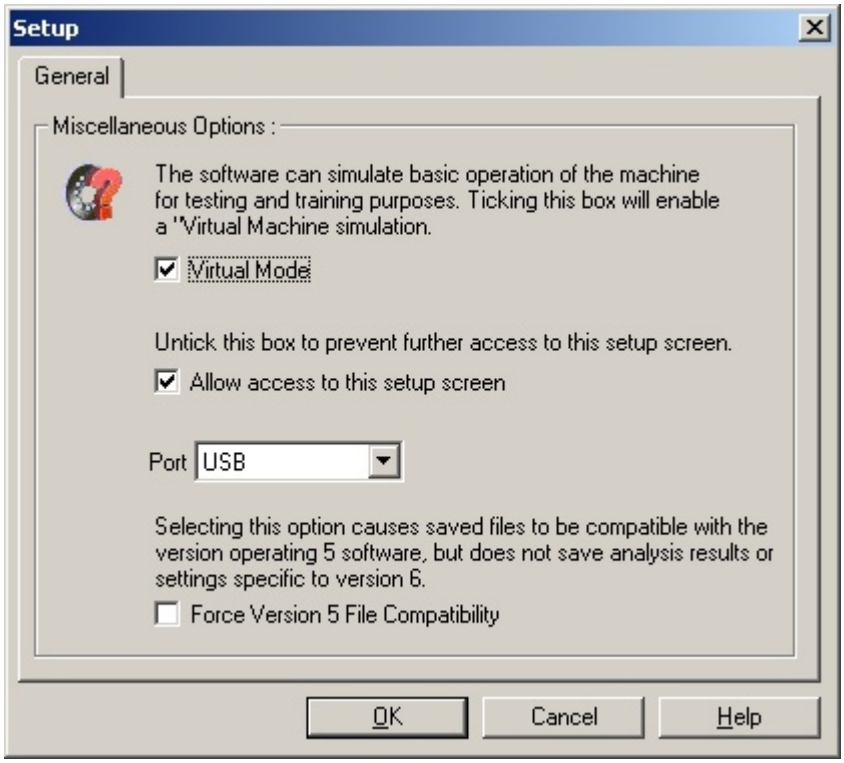
- Install the Rotor-Gene on an approximately level surface.
- Make sure there is enough clearance behind the unit so that the lid fully opens.
- Make sure you can reach the power switch at the back of the unit without difficulty.
- Before you turn on the Rotor-Gene, check the voltage selector switch at the back of the unit. This is set in the factory. Make sure the setting is correct for your local mains voltage. If it is not correct, change the setting and the fuse while the Rotor-Gene is unplugged from the mains. See the instrument's rear label for the correct fuse types.

  NOTE: If you change the voltage selector switch setting while the instrument is connected to the mains, you may cause damage to the instrument and/or blow the fuse. Always unplug the instrument from the mains before changing this switch setting.

- Connect the instrument to the PC using a standard 9-way serial cable. Plug one end of the cable into the 9-pin serial port at the back of the instrument. Plug the other end of the cable into a serial communications port on the PC, or into a USB port via a serial-to-USB adapter. Ensure that the correct port is selected in the Rotor-Gene Setup.

Software Installation
To install the Rotor-Gene software insert the Rotor-Gene software CD into the CD drive of the computer. The CD should open automatically. If not then browse to the CD drive. Double-click on the setup icon. Follow the setup wizard for quickest installation.

After connecting the Rotor-Gene to the power supply and the computer, switch on the instrument. The power switch is on the right side at the back of the machine. Double click the Rotor-Gene desktop icon to initiate the software. The **Rotor-Gene Setup** window will appear. Select the correct settings as described below.



Virtual Machine: Tick this option if the software is to be used without a Rotor-Gene attached. The software retains all functions. This mode is useful for demonstration purposes, for data analysis and setting up run templates.

Allow access to this screen: If this option is unticked during the setup process, subsequent access to this menu becomes severed. This security measure prevents users from altering the settings. To re-establish access you must contact your distributor.

Port: Select the correct Communication Port to enable communications between the computer and the Rotor-Gene.

Force Rotor-Gene 5 Compatibility: Tick this option if you want the run files to be compatible with version 5 operating software. Because not all new features in version 6 can be stored in version 5 data files, this option is only recommended if you need to interchange files between the two versions regularly.

2.4.2 Updating Software

Software updates are available of the Corbett Research web site, available from the Help menu in the software. To install the new software, browse to software download sections of the homepage. Download the Rotor-Gene setup file.

To initiate the installation double click on the setup file and follow the prompts. The previous version will be uninstalled and the new version automatically installed.

2.4.3 Warning Labels

The equipment has two warning labels; these have black symbols on a yellow background.

 The first warning label is a General Danger symbol near the mains voltage selection switch. Before you use the Rotor-Gene for the first time, make sure the mains setting is correct. See the Hardware Installation section above.

WARNING: If you change the voltage selector switch setting while the Rotor-Gene is connected to the mains, you may cause damage to the Rotor-Gene and/or blow the fuse. Always unplug the Rotor-Gene from the mains before changing this switch setting. See the Installation section above for details.

 The second warning label is a Hot Surface Warning label near the sample chamber; it is visible when you open the lid.

WARNING: DO NOT try to open the lid during an experiment, or while the Rotor-Gene is spinning. Otherwise, if you manage to overcome the lid lock and reach inside, you risk contact with parts that are hot, electrically live, or moving at high speed - you may injure yourself and damage the Rotor-Gene.

If you need to abort an experiment quickly, turn off the power to the Rotor-Gene, then open the lid. Let the chamber cool before reaching inside; otherwise you risk injury by touching parts that are hot.

2.4.4 Maintenance

The only maintenance the user may have to perform is keeping the lenses clean and free from dust that may build up over time. Dust build up in the excitation and detection sources (located in the bottom and side of the Rotor-Gene chamber) can result in the decrease of the fluorescence signal detected.

To avoid dust build up, keep the lid of the Rotor-Gene closed when the instrument is idle.

Clean the excitation and detection sources monthly. Use a cotton bud dampened with ethyl alcohol and gently rub the cover lens in a circular motion.

Chamber Decontamination

If the rotor chamber becomes contaminated with DNA, it can be cleaned by wiping the surfaces with lint free cloth dampened with a bleach solution. Do not drip bleach into the chamber.

After the bleach decontamination, clean the detection and excitation sources as described above.

2.5 Important: Read Before Running The Rotor-Gene

Before running the Rotor-Gene you should pay attention to the following:

1) DO NOT try to open the lid during an experiment, or while the Rotor-Gene is spinning. Otherwise, if you manage to overcome the lid lock and reach inside, you risk contact with

parts that are hot, electrically live, or moving at high speed - you may injure yourself and damage the instrument.

2) If you need to abort an experiment quickly, turn off the power to the instrument, then open the lid. Let the chamber cool before reaching inside; otherwise you risk injury by touching parts that are hot.

3) Always use a locking ring on the rotor. This will stop caps from coming off tubes during an experiment. Otherwise, if caps are free to come off during an experiment, they may damage the chamber.

Locking Rings: The 36-well locking ring is positioned over the tubes after the 36-well rotor has been locked into the chamber. The locking ring is held in place by the locking ring holder (see the section Locking Ring (36 Well Rotor)).To ensure that 0.1mL tubes sit firmly in the 72 well rotor, a 72 well locking ring is available. After loading the tubes, the locking ring can simply be placed into the rotor. (see the section Locking Ring (72 Well Rotor)).

4) If you touch the Rotor-Gene during an experiment, while you are charged with static electricity, then in severe cases the Rotor-Gene may reset. However, the software will restart the Rotor-Gene and continue the experiment

5) Ensure the software is not operating in the Virtual Machine Mode. Go to the **File menu** and select **Setup...** Check that the Virtual Machine box is not ticked.

If **Setup...** is not available, then it has been disabled by your distributor, who will have performed all necessary setup during installation.

2.5.1 Getting Started

The Rotor-Gene is set up using the following procedure:

1. Turn the desktop PC on and wait for Windows to launch.
2. Turn on the Rotor-Gene. The On/Off switch is at the back of the unit.
3. Double click on the Rotor-Gene icon.
4. The Rotor-Gene is now ready for use. The Wizard Selection screen appears, ready for run setup to commence. (See Setting up a Run for further information).

2.5.2 Software Version

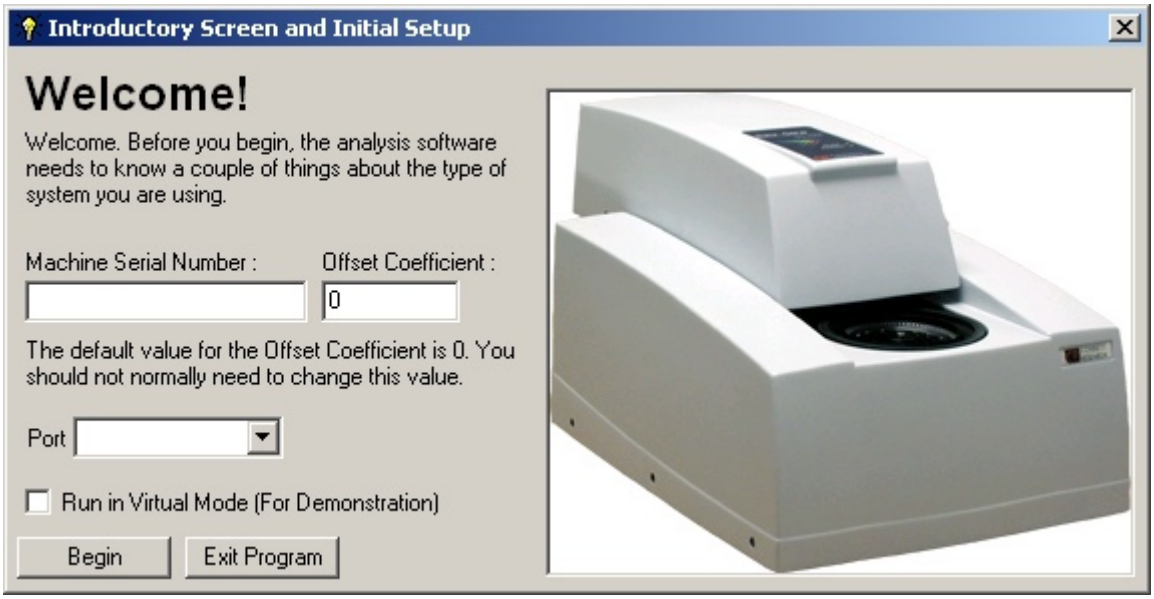
Software development for the Rotor-Gene system is ongoing. To check on your version number click on **Help** then **About Rotor-Gene...** The latest software version is available for download at our web site.

This screen displays general information about the software and specifically includes the version of the software, serial number of the machine, as well as the date it was last updated.

The software may be freely copied for use within an organisation owning a Rotor-Gene. The software may not be copied and distributed to others outside the organisation.

2.5.3 Welcome Screen

This screen appears when the Rotor-Gene software has been installed on a computer for the first time and the Rotor-Gene icon is double clicked for the first time after the installation. This screen does not appear when software is upgraded.



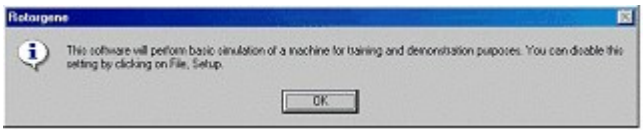
Machine Serial Number: Type in the serial number (six digits), which is located at the back of the Rotor-Gene.

Offset Coefficient: Type in the Offset coefficient, which can be found next to the serial number of the machine. If no number can be found, the value 0 should be entered.

Run in Virtual Mode (for demonstration): Ticking the box allows installing the Rotor-Gene software on a computer without Rotor-Gene attached. The software is fully functional and can even simulate runs.

NOTE: If this box is ticked with a Rotor-Gene connected to your computer, a message will appear before you start your run: "*You are about to run in Virtual mode*". To be able to perform a "real" run, the setup (see setup) has to be changed.

Begin: When all parameters are set, press Begin. A window will come up initializing the machine. Wait until the machine is initialized, which might take a few seconds. If virtual mode was chosen the following screen appears:



If the Run in Virtual Mode box stays unticked the machine is initialized and opens up the Rotor-Gene software automatically.

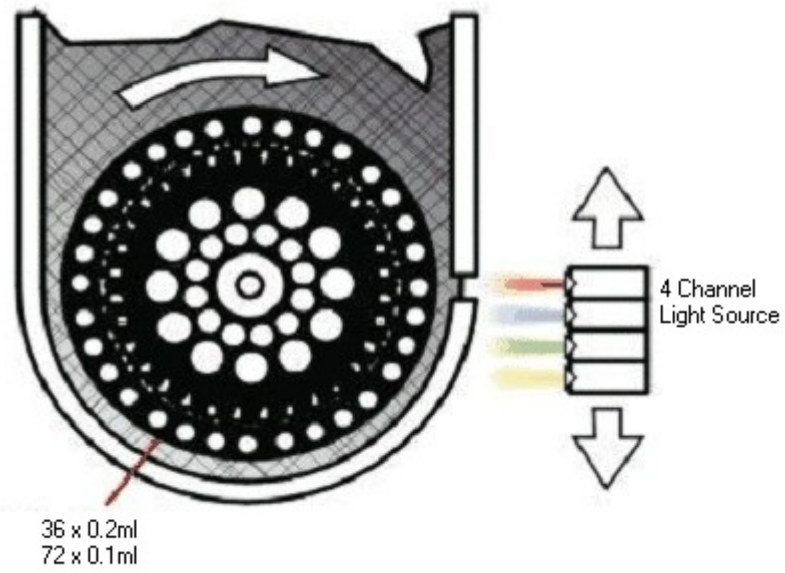
Exit Program: Exits program.

3 Instrument Description

3.1 Overview

The Rotor-Gene has four excitation sources, positioned on the side of the chamber. The four LEDs are coupled with narrow bandwidth filters. The filters for the excitation sources are: 20 nm bandwidth 470 nm filter, and 10 nm bandwidth 530, 585 and 625 nm filters.

The Rotor-Gene 3000A is similar having only two excitation sources. The filters for this model are the same as the Rotor-Gene but they do not have 585 and 625 nm filters.

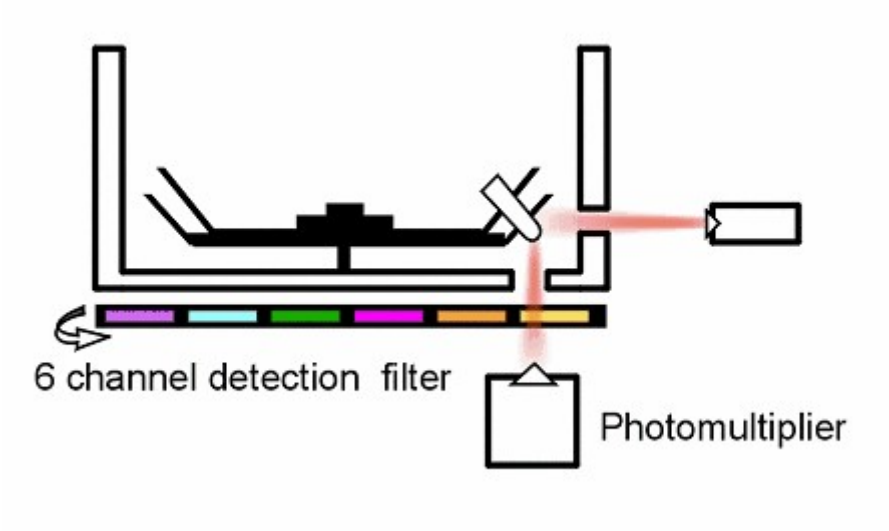


Chamber (Top View)

From the side view of the heating/cooling chamber we see the LED source illuminating the tube from the side wall and the photomultiplier (PMT) detecting the energy from the base of the chamber. For the Rotor-Gene the detection filter wheel has six filters, three 10nm band-pass (510 nm, 555 nm and 610 nm) and 3 high pass filters (570 hp, 610 hp and 665 hp). The high pass filters allow the instrument to detect fluorophores that cannot be detected using a bandpass filter. The instrument has four preset channels that are used in multiplex runs: FAM (470/510 excitation/detection), JOE (530/555 excitation/detection), ROX (585/610 excitation/detection) and Cy5 (625/665hp excitation/detection).

The Rotor-Gene 3000A is similar having only three filters, two 10nm band-pass (510 nm, 555 nm) and one high pass filter (610hp). This model has two preset channels FAM and JOE.

NOTE: These channels are not limited to detecting only the fluorophores used in their names. Other dyes that have spectra similar to these may be detected in the same channel, e.g. VIC, HEX and Yakima Yellow can be detected in the JOE channel.



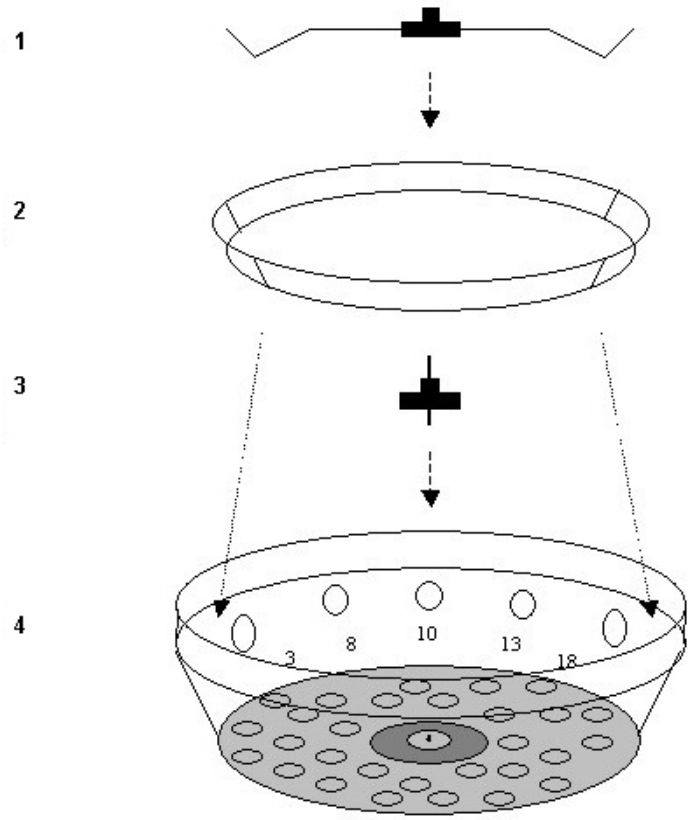
Instrument Chamber (Side View)

3.2 Locking Ring (36 Well Rotor)

Opening and closing 0.2 tubes several times before running might loosen the tube seal, resulting in tubes opening during cycling. The 0.2 tubes used in the 36 well rotor on the instrument should only be closed once before running. **Also, make sure that the 0.2 ml tubes are closed tightly by firmly pressing down the lid of the tube.** To avoid tubes opening during a run use the 36-well rotor locking ring. **The Locking Ring is designed to use flat top 0.2 ml tubes only.**

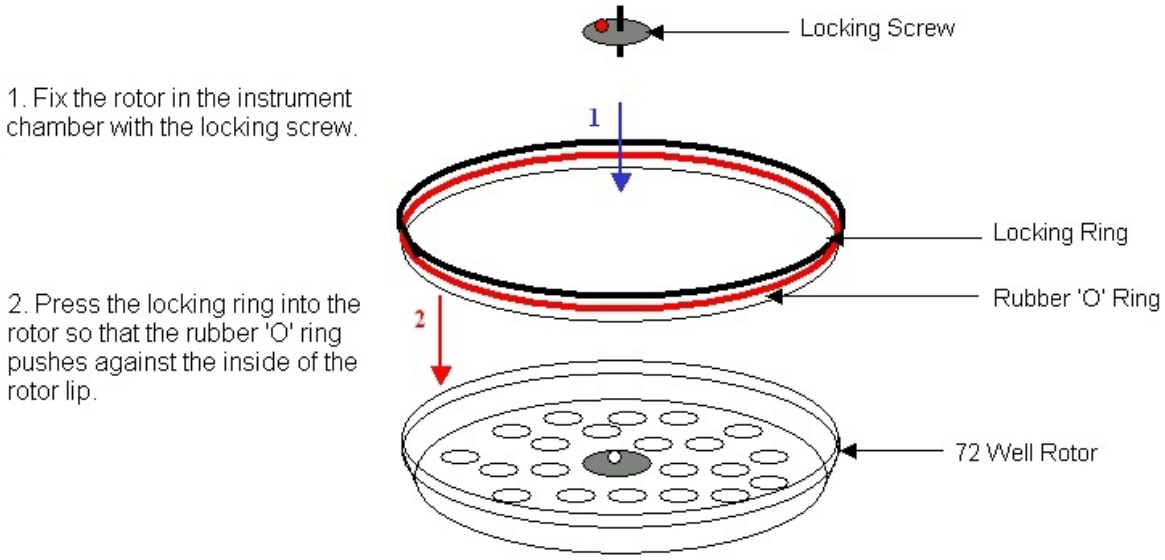
The graph below schematically shows how the locking ring must be used:

1) The 36-well rotor.
2) Tighten the screw with threads on both sides. Make sure the middle raised area faces the top.
3) Simply place the locking ring over the top of the tubes.
4) Screw down the locking ring holder (4) over the screw with threads on both ends (2) until the locking ring (3) is fixed.



3.3 Locking Ring (72 Well Rotor)

As with the 0.2 ml tubes, the lids of the 0.1 ml tubes may detach from the vessel during a run if they are not fixed tightly. The 72 well Locking Ring should be used to prevent this.
To use the locking ring, simply place the tubes in the rotor, place the rotor in the Rotor-Gene chamber and secure with the Locking Screw. Place the Locking Ring in the rotor so that the rubber 'O' ring of the Locking Ring pushes against the inside of the rotor lip. The lids will be secure and will not detach from the tubes.



4 Two Different Rotor Systems (Tubes)

4.1 Rotor 36 Well System

It is recommend that you use flat cap 0.2 ml tubes from Axygen, supplied by Corbett Research.

If using other tubes, be aware of the following:

1. The tube cap must close the tube tightly, otherwise, there is a risk that the cap may 'pop' open during thermal cycling. Use the locking ring to secure the tubes.

2. Be sure that the tubes fit into the rotor firmly. Tubes that are excessively loose may vibrate during a run interfering with data acquisition and give poor results.

3. Ensure that the tubes are not too wide. If the diameter of the tube is too large it will not sit inconsistently in the rotor. Samples will not be optically aligned over the detection system and may result in a reduction in fluorescence signal acquired and sensitivity.

4. Do not use tubes from different manufacturers within the same run. This may result in inconsistencies.

5. Locking Ring can only be used with **flat top** tubes.

4.2 Rotor 72 Well System

Corbett Research manufactures the 72-well tubes and caps in strips of four specifically for the Rotor-Gene.

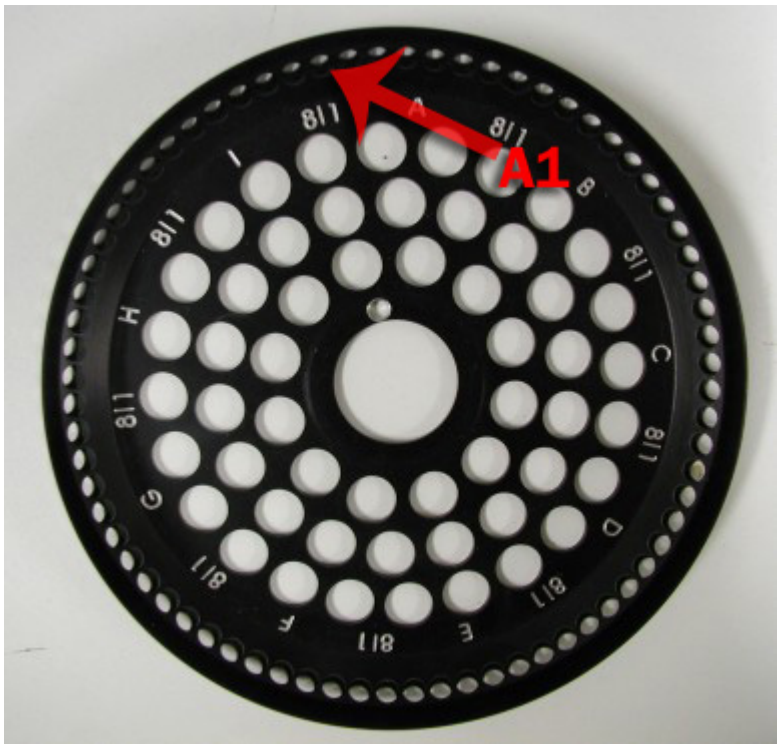
DO NOT autoclave the 72-well tubes or strip caps as this may distort the tubes and caps.

The tubes can become visibly deformed during the autoclaving process. When running tubes that have been autoclaved the caps may detach from the tube.

Ensure that the 72-well Locking ring is used when running this rotor. Loose caps in the heating/cooling chamber can damage to the insulation material.

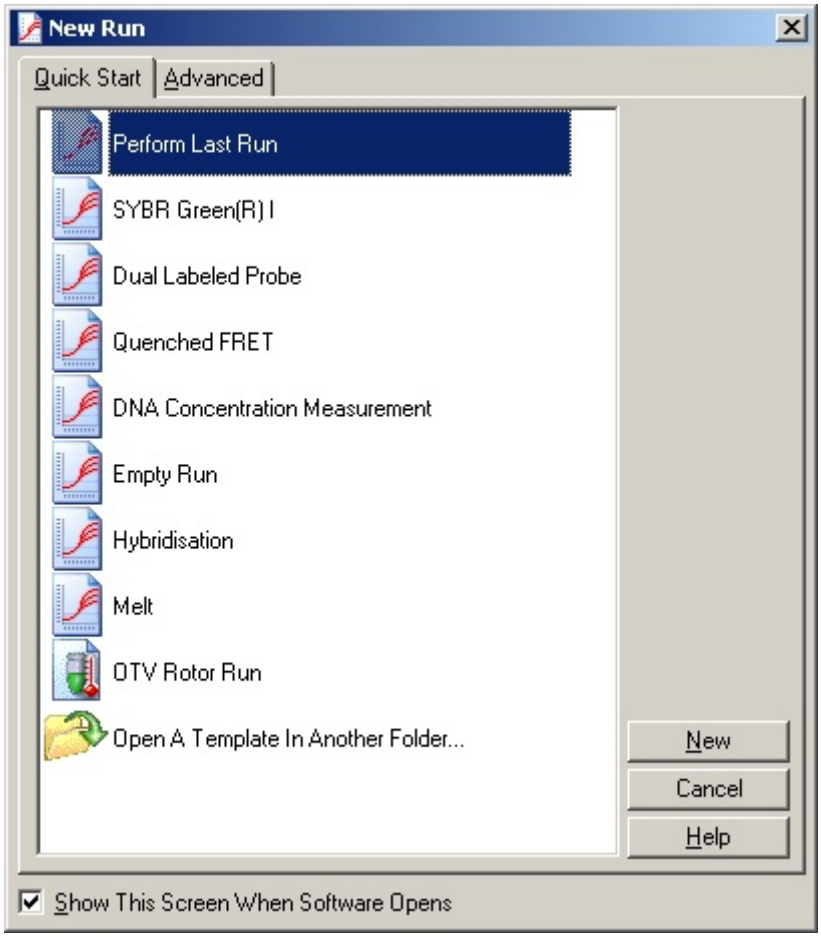
It is not necessary to cap empty tubes that are only used to fill up the rotor.

The 72-well rotor, with the A1 position highlighted, is shown below:



5 Setting up a Run

New runs can be set up using the concise Quick Start or the Advanced wizard that contains the gain adjustment option. For convenience the wizards contain a number of templates which have default cycling conditions and acquisition channels.



Perform Last Run imports the cycling and acquisition and sample definitions from the last run open in the software.
SYBR Green (R) I: three step cycling profile and a melt curve with data acquisition on the FAM/SYBR channel.
Dual Labeled Probe: two step cycling profile with data acquired on all channels.
Quenched FRET: three step cycling profile with and a melt curve, data acquired on the FAM and JOE channels.
OTV Rotor Run is used with Corbett Research's Optical Temperature Verification rotor.
DNA Concentration Measurement is a default template for measuring the concentration of nucleic acid using intercalating dyes.
Demo Kit contains profiles used for the SYBR Green I ® and Dual Labeled Probe demonstration kit.
The cycling and acquisition profiles can be altered during the wizard for all templates except the **OTV Rotor Run** and **DNA Concentration Measurement.**

User defined templates can also be added to wizards.

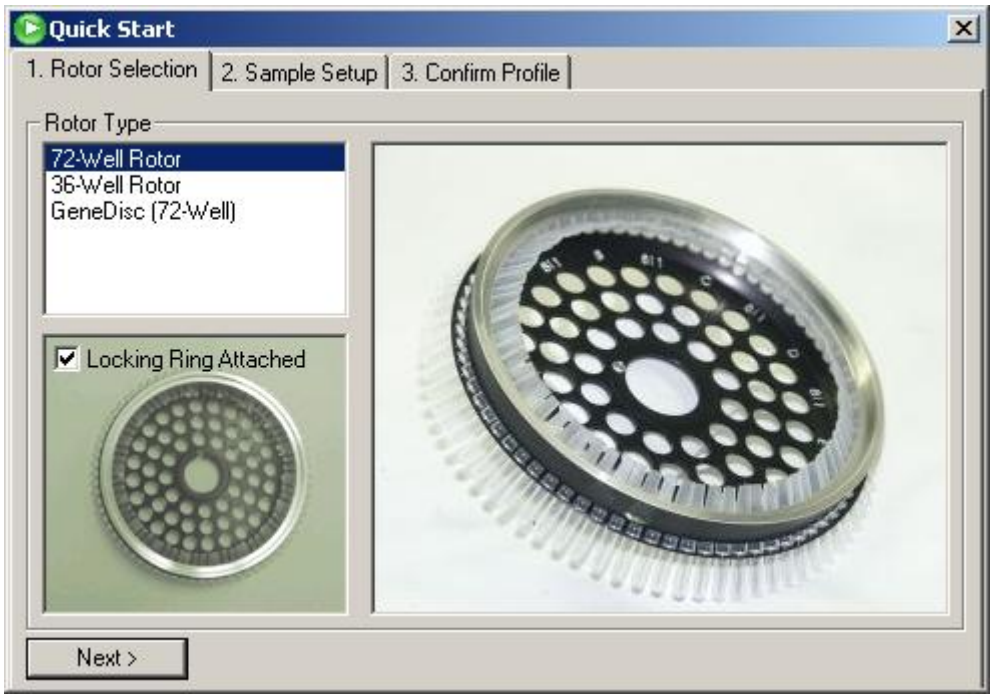
5.1 Quick Start Wizard

The first step is to select the desired template for the run. Select from the available templates, as described in the previous section.

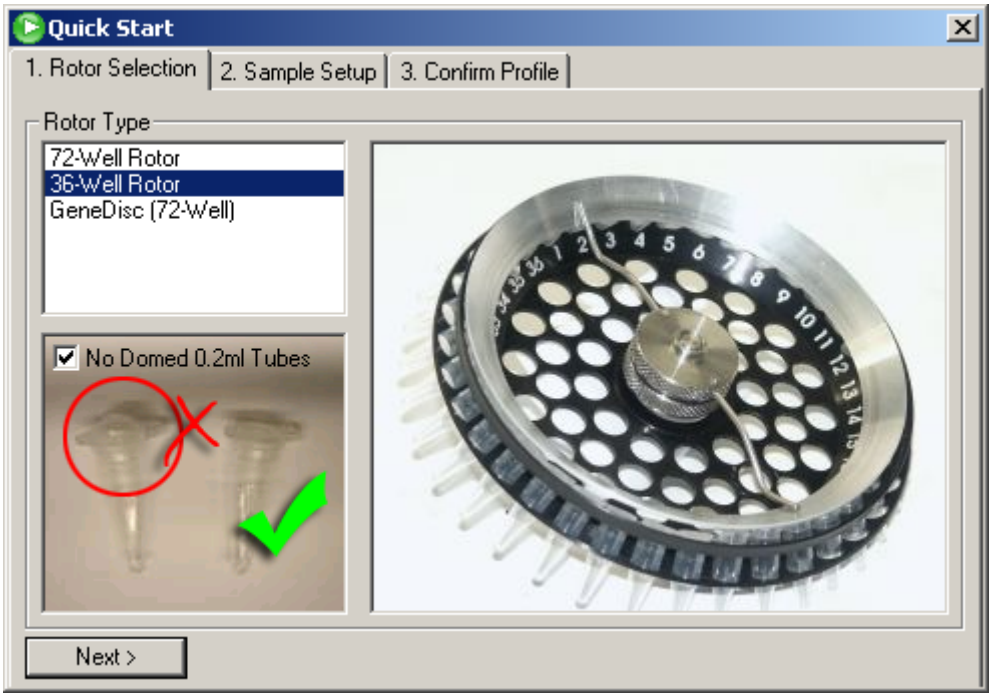
5.1.1 Rotor Selection

Select the rotor type.

If a 72-Well Rotor or a GeneDisc (72-well) is selected, the locking ring for the rotor must be attached to the rotor. Tick the **Locking Ring Attached** option to proceed through the wizard.

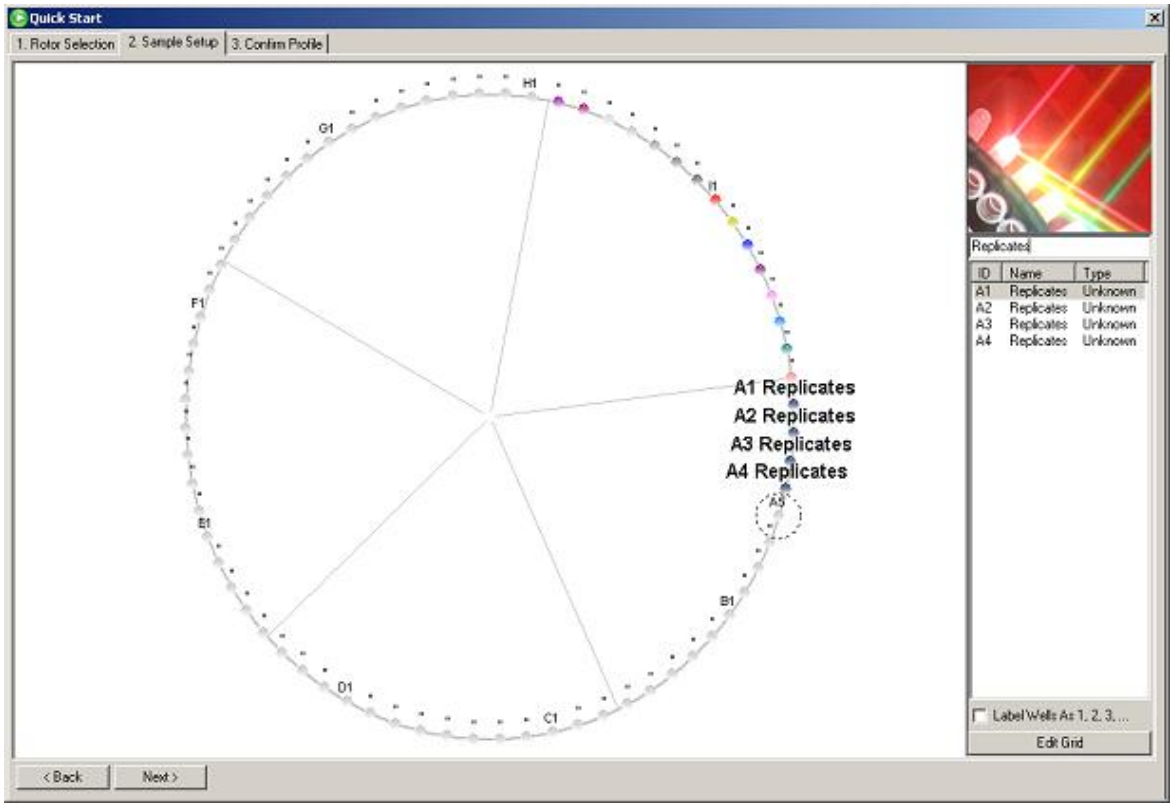


It is recommended that flat capped tubes be run on the Rotor-Gene. When using these tubes, tick the **No Domed 0.2 ml Tubes** option to proceed through the wizard.



5.1.2 Sample Setup

Enter sample names. By default all positions in the rotor are designated the name and type of **Unknown**. To change the name and to designate rotor positions as replicate reactions use the mouse to select the rotor positions of replicates in the graphic and enter the same name. The software will recognize these wells as replicates.

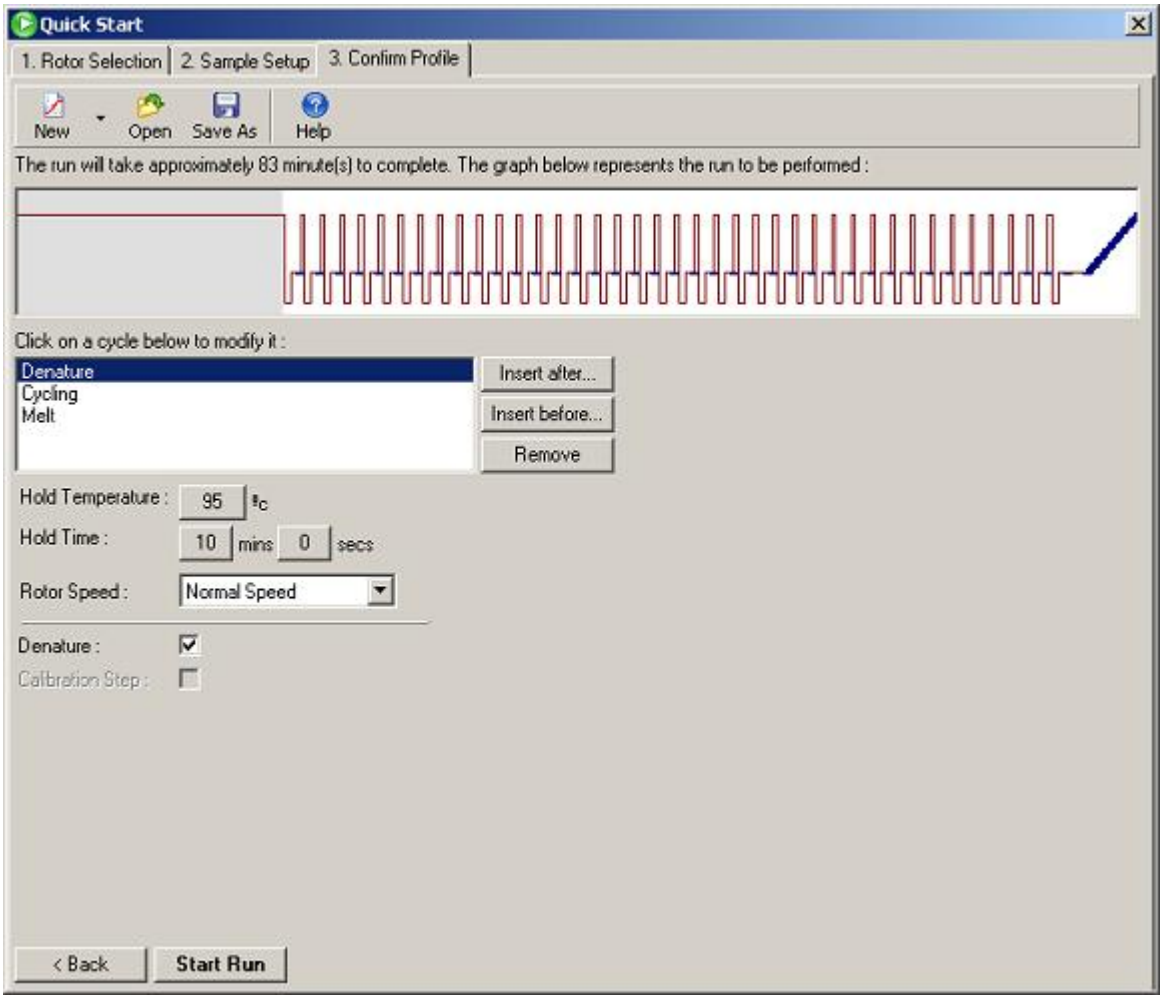


Click on the Edit Grid button to view sample setup information in a tabular format. This format provides more options for editing sample inforamtion, such as setting the known concentration of each standard, for example.

Note: It is not necessary to complete the sample setup information before the run is started. You can skip this wizard step and complete the information later.

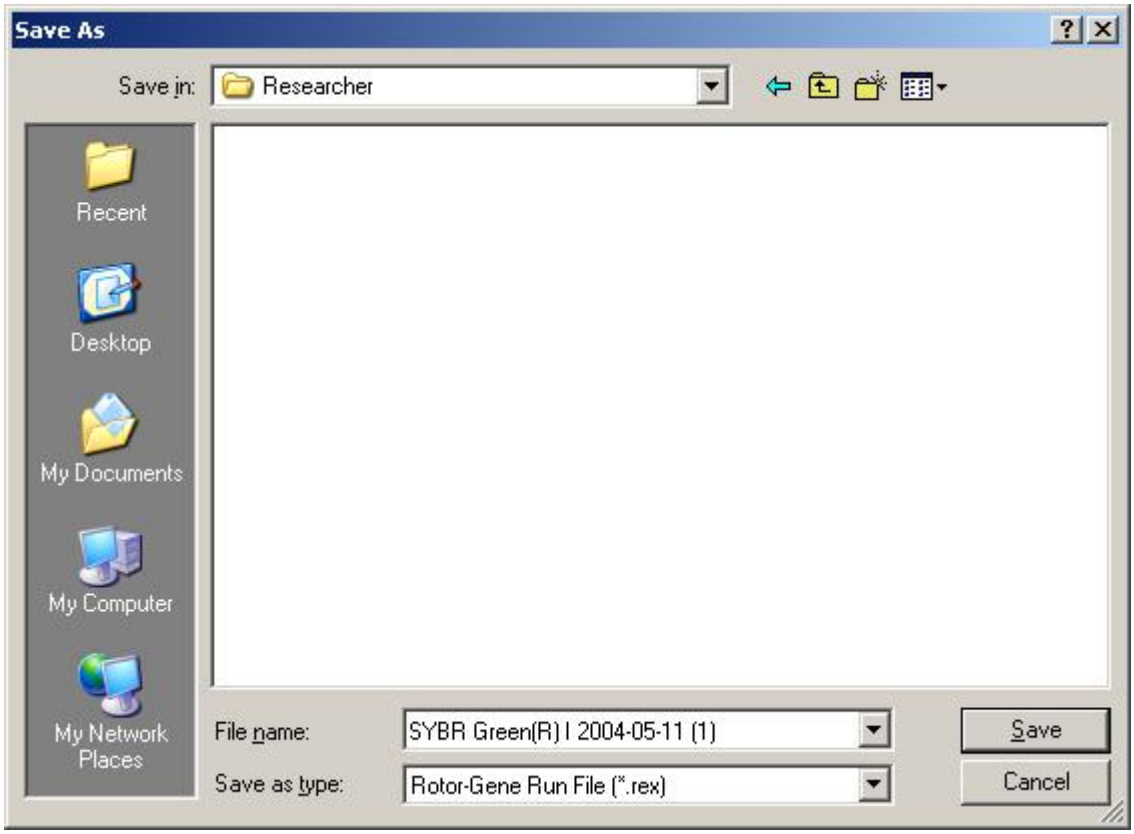
5.1.3 Confirm Profile

The template chosen imports the cycling conditions and acquisition channels. This can be altered using the Profile Editor. To initiate a run click the **Start Run** button.



5.1.4 Save Run

Clicking the Start Run button brings up the Save As window. The run can be saved in the user's desired destination. The run is given a filename with the template used and the date of the run. A serial number is also designated to allow automatic naming of numerous runs that use the same template on the same day. E.g. below the run was the first run set up using the SYBR Green (R) I template on 11'th May 2004.

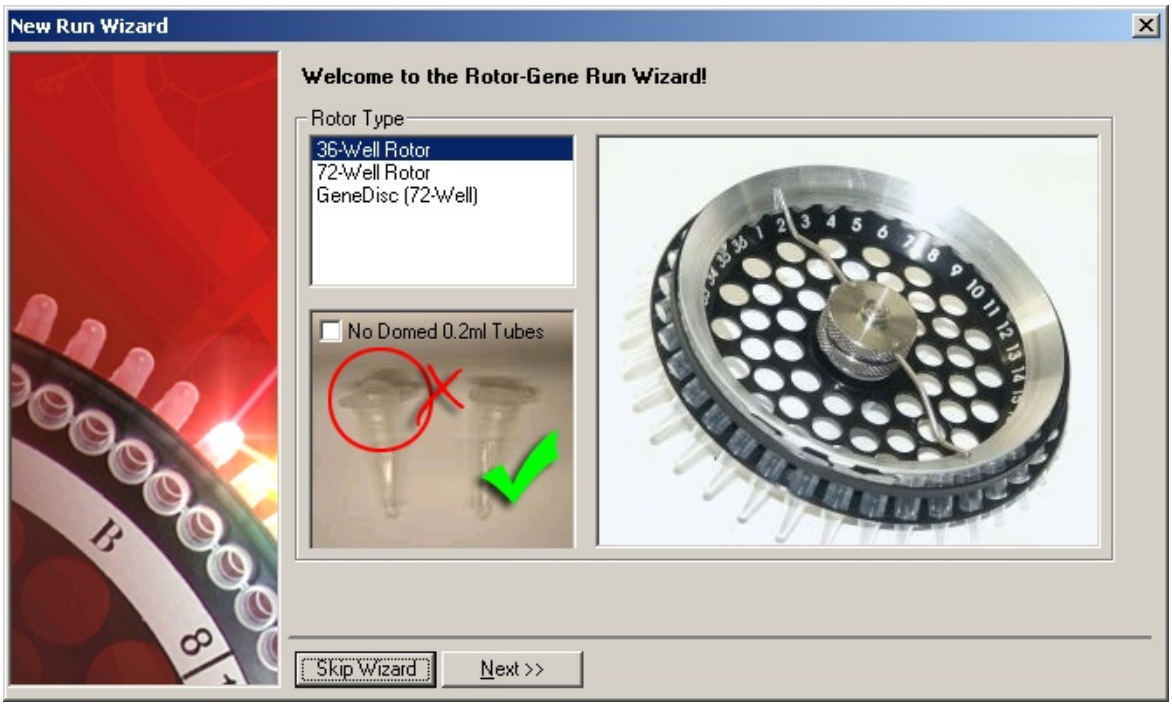


5.2 Advanced wizard

Select a template from the Advanced tab of the wizard (see the topic Setting up a Run). The advanced wizard contains options that are not available in the Quick Start wizard such as selecting the rotor speed and gain adjustment.

5.2.1 Welcome Screen

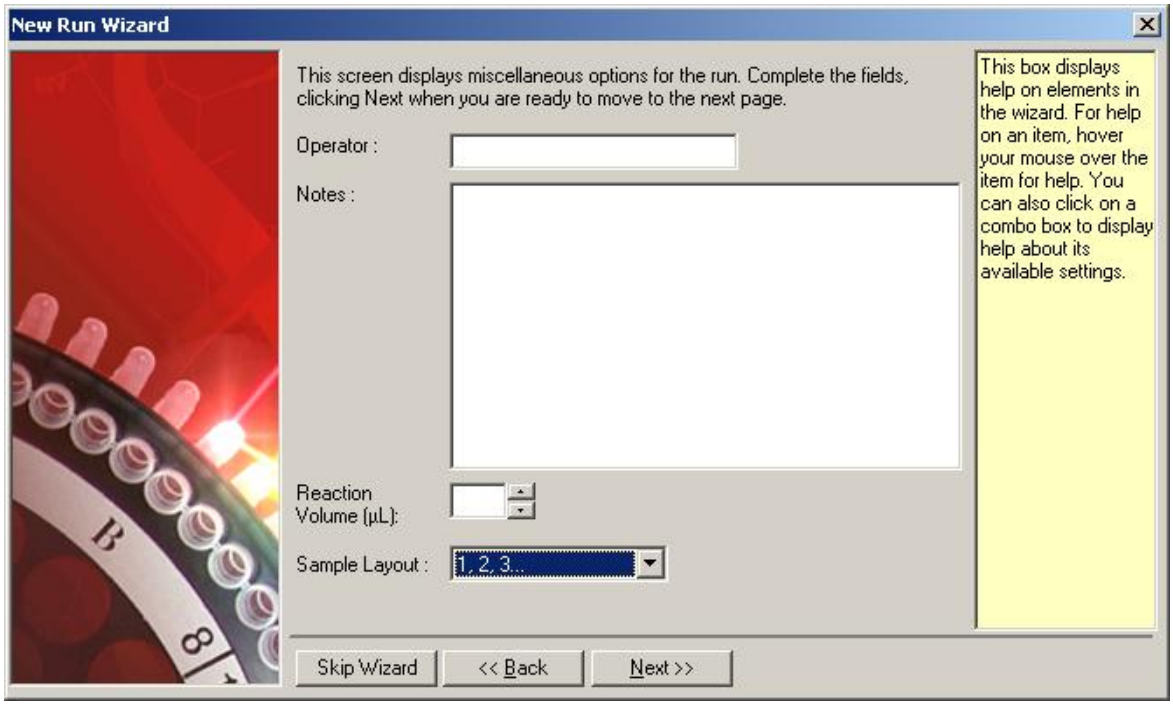
The initial screen on the new run wizard asks for the selection of a rotor. As in the Quick Start Wizard, you must acknowledge the displayed warning before continuing.



5.2.2 Page 1

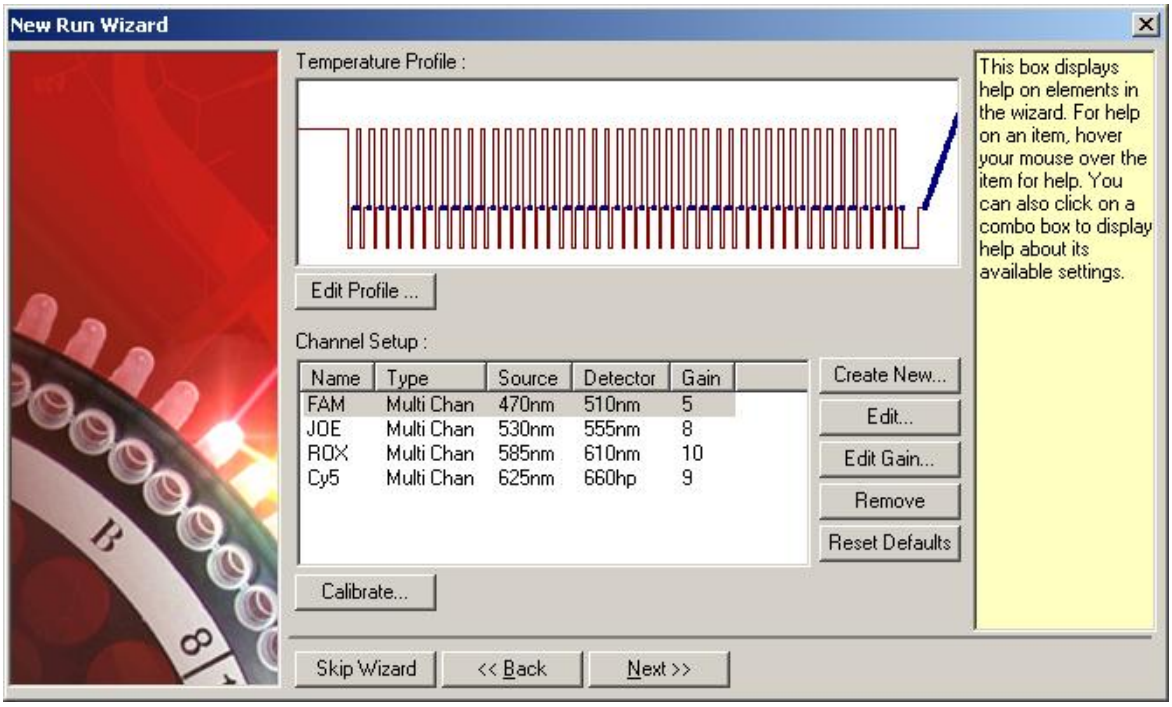
Operators name and notes about the run can be entered here. The reaction volume must be entered. The Rotor-Gene is configured for 20-25 µL volumes. The software recommends longer holds during cycling for larger volumes.

If the 72 well Carousel is selected, three **Sample Layout** formats are available. **1, 2, 3...** should be selected when a numbered rotor is used, this is the default option. **1A, 1B, 1C...** when samples are loaded in adjacent tubes in the 0.1mL strips. If consecutive samples are in fact in every 8'th well (if loaded with a multichannel pipette) the **A1, A2, A3...** layout should be selected. Most users will only need to use the default option.



5.2.3 Page 2

In this screen the **Temperature Profile** and the **Channel Setup** can be modified. Click the **Edit...** button to bring up the Profile Editor to alter the cycling conditions and select acquisition channels. After setting up the profile click the **Calibrate...** button to bring up the Gain Adjustment window.



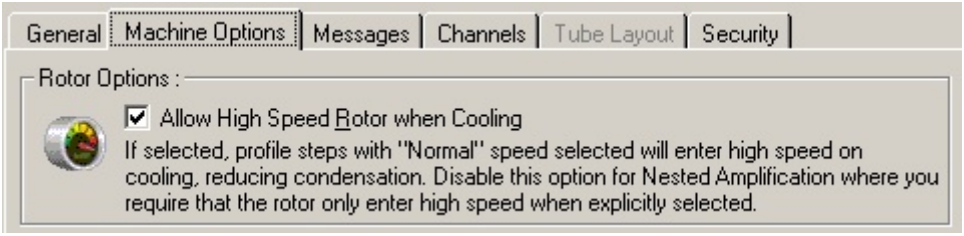
Profile Editor

The Profile editor allows you to set the cycling conditions and acquisition channels. The initial cycling profile is based on the template selected (Setting up a Run). The profile is graphically displayed. The list of the segments that the profile consists of is listed below the graphical display. A cycle can be a **Hold**, **Cycling** or a **Melt**. The settings of each cycle of the profile can be edited by clicking on the cycle in the graphical display or the name of the cycle in the list and changing the set temperatures and hold times.

Insert after...: allows addition of a cycle after the selected cycle.
Insert before...: allows addition of a cycle before the selected cycle.
Remove: removes the selected cycle from the profile.

Rotor Speed: The rotor speed can be selected for any hold except where data is acquired. The choices in the drop down menu are **Normal Speed**, **Still Rotor (Incubation)** and **High Speed**. Unless you have a special application, it is recommended to leave this setting on **Normal**.

By default, the Rotor-Gene will operate at normal speed while heating and holding, and at high speed during cooling. This is an optimal configuration as the high speed improves cooling times and can reduce condensation. This mode of operation is configured by the "Allow High Speed Rotor when Cooling" checkbox inside the Machine Options tab of the Run Options window.



For specialised applications, you may wish to directly control the rotor speed. To do so, first disable the "Allow High Speed Rotor when Cooling" option. All heating, cooling and hold operations will be performed at normal speed. Once set, you can then configure rotor speeds for each step. Not all combinations of instrument operation and rotor speeds are possible. For example, the rotor must be at normal speed while acquiring data.

Cycling

Long Range option adds 1 second to the hold time at extension at each cycle for a user defined number of cycles.
Touchdown decreases the temperature at annealing by 1 degree at each cycle for a user defined number of cycles.

Melt and Hybridisation

A **Melt** step is a ramp between two temperatures, from a lower to a higher temperature. If the temperature is set to decrease, the step is labelled as **Hybridisation** instead.

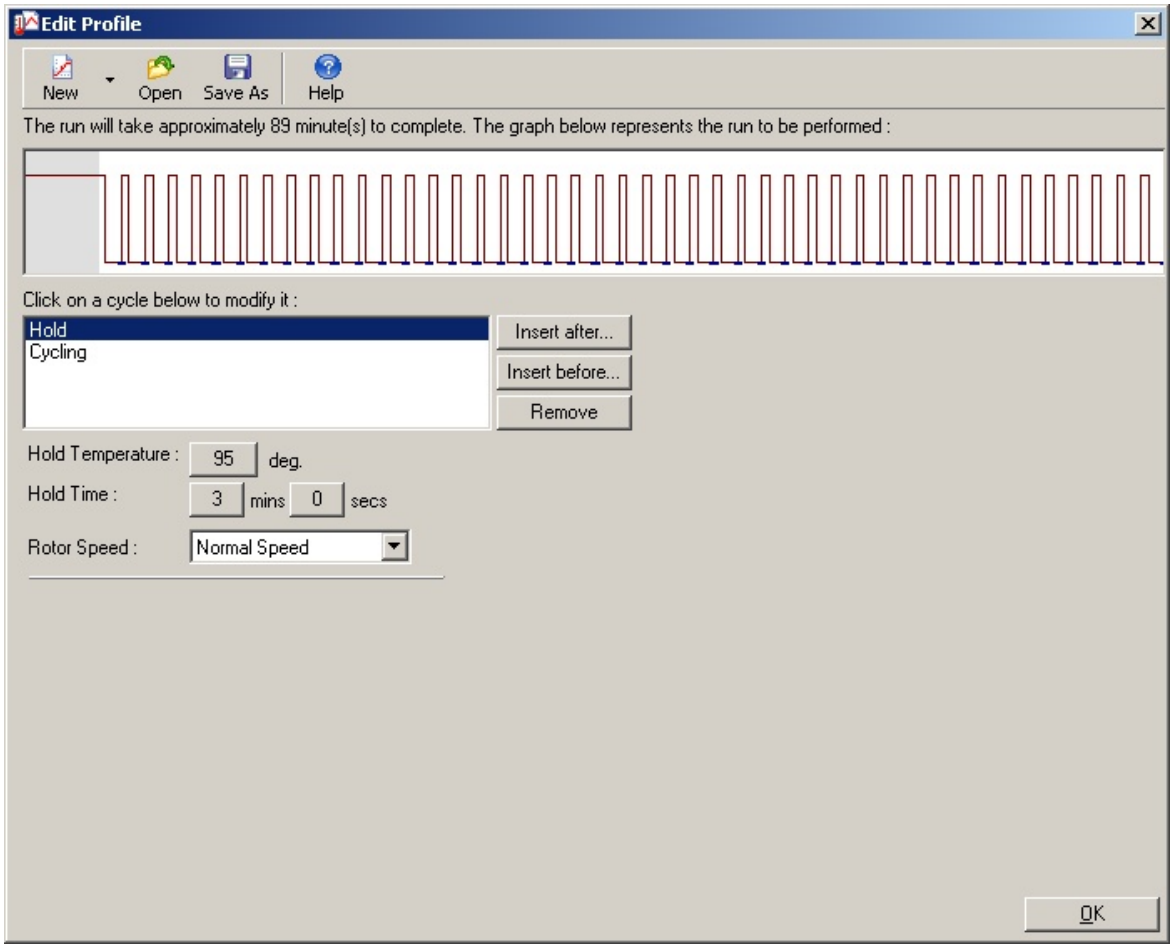
To set up a melt, define the start temperature, the end temperature, the length of time to hold at the first acquisition temperature before the ramp is initiated, the temperature increments and the time each increment is to be held for, and the acquisition channels.

Hold

A hold instructs the Rotor-Gene to remain at the designated temperature for a set time. To change the temperature click **Hold Temperature** button and use the type in or use the slide bar to change the temperature. Similarly to change the duration of the hold click on the **Hold Time**, **mins** and **secs** buttons.

If running an Optical Denaturation, you can elect a hold step as a calibration step. This causes a calibration melt to be performed before this step. By default, this is configured for the first hold in the run, but may be overridden if you are using 60 degree preholds before your 95 degree hold.

More information about configuring optical denature runs can be found in the section Optical Denature Cycling.

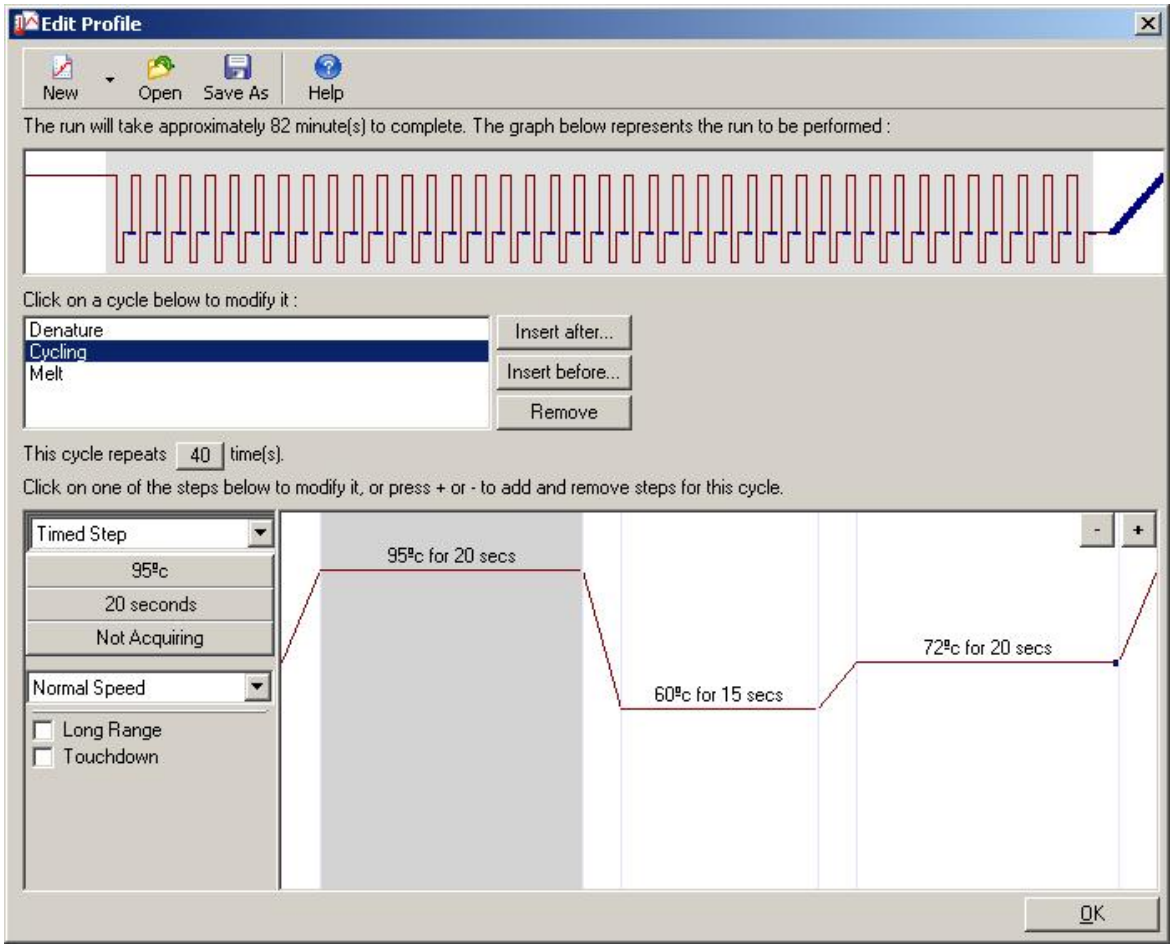


Cycling

Repeats the user defined temperature/time steps a defined number of times. The number of repeats is set using the **This cycle Repeats X time(s)**.

The individual repeat is displayed (as seen in bottom right of the below graphic). Each step of the repeat can be altered. Temperature can be changed by dragging the temperature bar up or down. The duration of the step can be changed by dragging the temperature boundary left or right. Alternatively click on the step and use the temperature and time buttons to the left of the repeat display.

Steps can be added or removed from the cycling by using the -/+ buttons on the right-hand side.



Touchdown: Touchdown can be enabled to decrease the temperature during the initial cycles. Set the number of cycles and the program decreases the Anneal Temperature by 1°C every cycle for the number of cycles specified. This is reflected in the graphical cycle representation.

Long Range: Long range can be enabled to increment the hold time of the selected step by one second with each new cycle. This has the effect of increasing the extension time as the cycling proceeds.

Acquisition

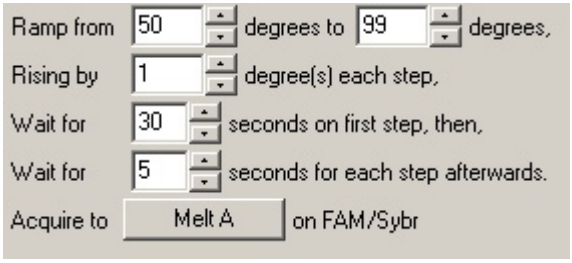
Acquisition: Data can be acquired on any channel at any step of the Cycling. Click on the **Not Acquiring** button. If a channel has been already been set to acquire at this step, then the acquiring channels will be listed here instead. Once clicked, the Acquisition window will appear. To set a channel to acquire, move the channel from the **Available Channels** list to the **Acquiring Channels** list using the [>] button. To remove a channel from the acquiring channels list, use the [<] button. The [<<] button removes all the channels from the acquiring channels list. Clicking the Don't Acquire button also removes all acquisitions from the step.

If more than one Cycling cycle is included in the profile, the acquired data can be appended to the data acquired from the earlier Cycling. Use the drop down menu in the **Same as Previous** option to select the cycling step to which the data is to be appended.

The acquisition options described above equally to Melt steps, except one cannot chain acquisitions together using the **Same as Previous** option.

Melt and Hybridisation

For a melt cycle, specify a start temperature, an end temperature, the time you want to wait at before the first acquiring point, the amount of time to remain at each point and a ramp will be generated going between the two temperatures. If the start temperature is higher than the end temperature, the name of the step will change to **Hybridisation**. The **Acquiring To** option, here set to **Melt A**, can be changed by clicking on the button. The same screen as for Cycling will appear and the channels to acquire to can be selected.



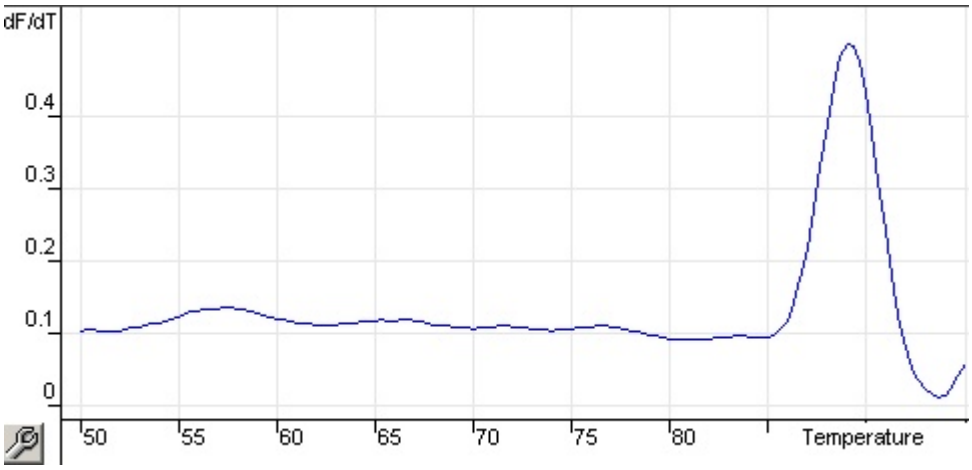
When running a standard melt the temperature is increased by increments of 1ºC. The Rotor-Gene can be configured to perform melts in 0.1ºC increments.

Optical Denature Cycling

What is Optical Denature Cycling?

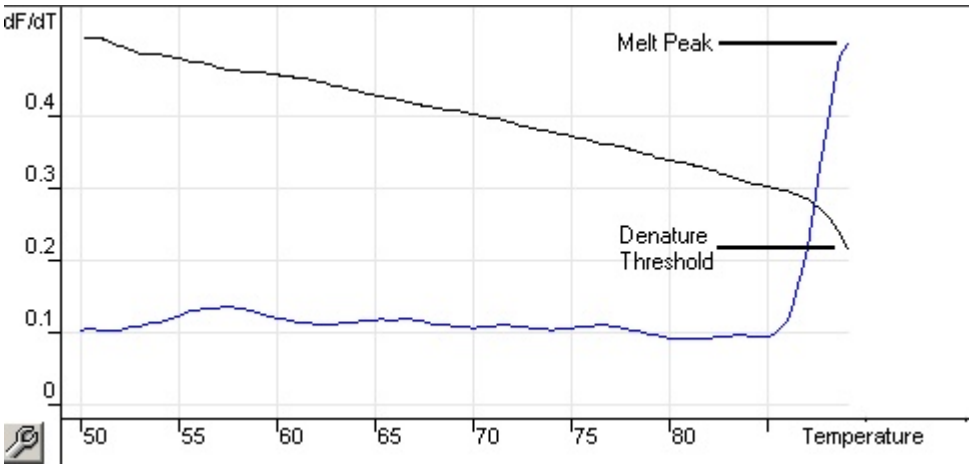
Optical Denature Cycling is an exciting new technique, available only on the Rotor-Gene, which performs real-time melt analysis to determine the melt peak of a reference sample. This indicates with greater precision when your product has denatured than by setting a particular denature temperature for a hold time. To perform this technique, simply place a tube of pre-amplified product in tube position 1 in your rotor. The reference tube must also contain a detection chemistry that enables strand dissociation to be detected. We suggest using SYBR Green I.

When first heating the machine to the initial denature temperature, a melt on FAM/SYBR is performed starting from around 80 degrees until 95 degrees (although these parameters may vary). From this data, a melt curve is generated and automatically analysed:



The **Melt Peak** is referenced back to the raw data to obtain a **Denature Threshold**. Then, every Optical Denature step, the machine is heated as quickly as possible and data acquired continuously. Once the **Reference Tube** has reached this denature threshold fluorescence level, the machine is immediately cooled and proceed to the anneal step. While cycling, a peak is not calculated, rather, it is the fluorescence level that is referenced to the melt peak and designates the denature threshold.

In this graph, the raw fluorescence readings and the first derivative have been overlayed. It shows the correspondence between the **Denature Threshold** and the **Melt Peak** obtained during the calibration.



What do I need to perform an Optical Denature run?

To perform an Optical Denature run, you will need:

- A pre-amplified sample which you should place in position 1 in the rotor. This sample should contain the same product as samples of interest and a detection chemistry, such as SYBR

Green, that allows for the monitoring of product dissociation.
- An optical denature profile. You can find out how to set up an Optical Denature Run in the topic Adding a New Optical Denature Cycling Segment.

What will I see when performing an Optical Denature run?

An Optical Denature run, from the user perspective, appears almost identical. The most striking differences are the melt step automatically inserted at the beginning of the profile, and the sharp profile of the denature step during cycling. The denature step does not require defined hold times as the dissociation of the product is monitored at each cycle.

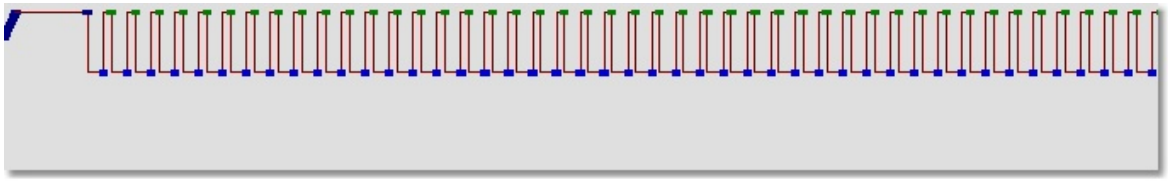
Configuration

To perform this technique, the software must know several things about your run:
- The Initial Denaturation temperature. This is the same temperature as your denature step in a standard cycling profile.
- The tube position of a sample which has already been amplified, and which will produce a melt curve on the FAM/SYBR Channel. For example, if you are performing a SYBR-Green I run or a run with dual labeled probes, put a sample of pre-amplified SYBR product in tube position 1, and your other samples in the remaining positions.

You must also define an Optical Denature cycling profile. This is enabled by default in this version of the software. However, your existing templates from older versions of the software will not use this step.

Adding a New Optical Denature Cycling Segment

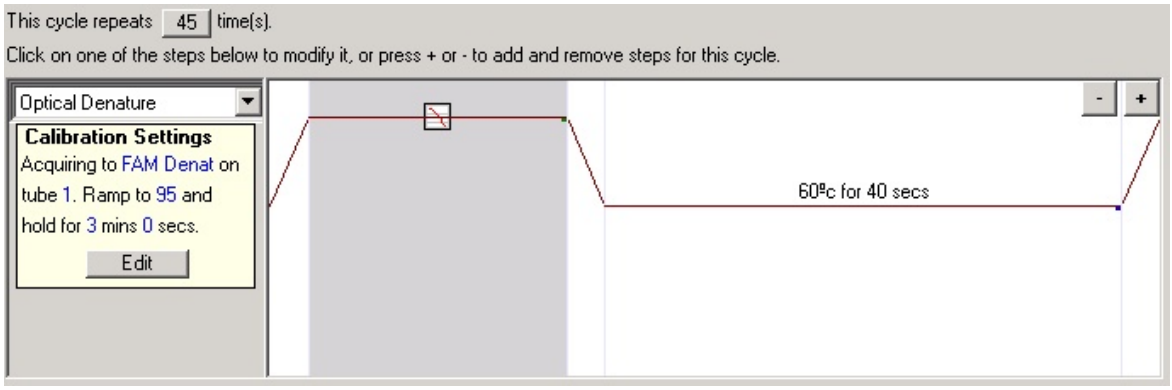
To add an optical denature step, open the Profile Editor. Then click New. A default profile containing a Denature step and an Optical Denature Cycling step will appear: The profile is displayed as:



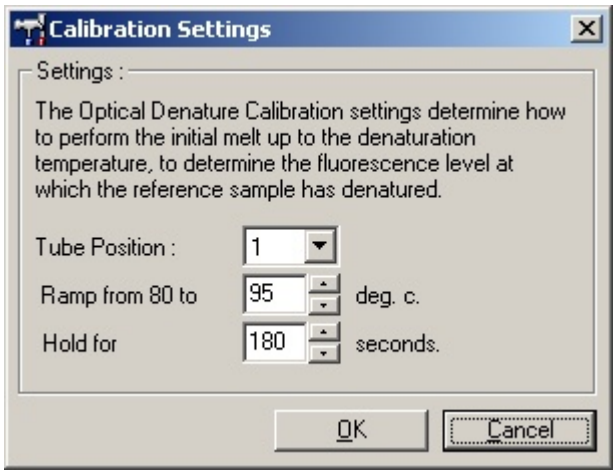
The blue shaded region at the beginning of the run represents the optical denature calibration process. The Green dots represent the acquisitions taken each cycle during heating. The lighter blue dots represent the acquisition at the end of the anneal step at 60 degrees. Note that while the profile shows each step going to the same denature temperature, this may not be the case. If the sample requires slightly longer to melt towards the end of the run, the optical denature process will wait for the melt in the fluorescent data, and not off the times. For this reason, the temperature trace for optical denature may vary for each cycle.

By clicking on the Cycling step, the information about the 2-step profile appears in the lower half of the Profile Editor window. Click on the second half of the graph with the Optical Denature symbol  .

The Calibration Settings information appears on the left-hand side of the screen:



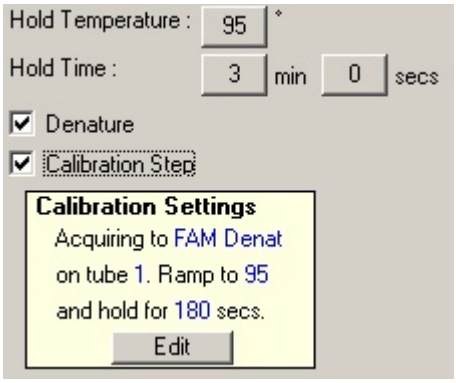
Most of the time, this information will be correct. You can, however, modify it by clicking **Edit**. The Calibration Settings window will then be displayed:



You should ensure that:

- The tube indicated in the Tube Position contains a sample of pre-amplified product that will show a melt peak on the FAM/SYBR channel.
- The Final Ramp temperature will not burn the sample, yet will be high enough to allow it to melt.
- The hold time is sufficient to denature the samples.

You can also define a Denature step yourself, and configure the calibration settings via its screen:



The calibration settings are synchronised with the denature settings, so a change to the hold time in the denature step will automatically update the calibration's hold time. This is because the calibration process and denaturation are equivalent in **Optical Denature Cycling**.

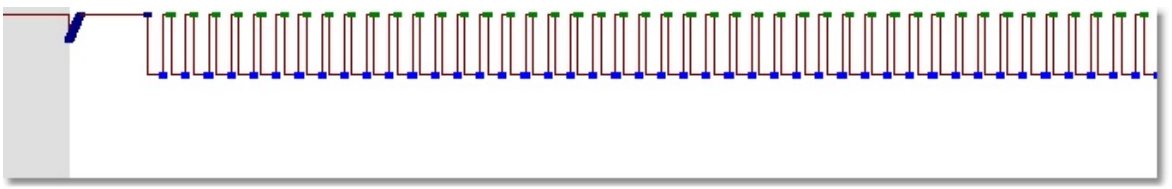
Changing An Existing Step to Use Optical Denature

To change an existing denature step in a Cycling, select the cycle in the Profile Editor's list. Then, select the denature hold by clicking on it in the preview graph at the bottom of the screen:

Click on the word "Timed Step" and select "Optical Denature". The Temperature and Hold Time will be removed and the Optical Denature icon will be displayed in their place.

IMPORTANT NOTE:

- If you are using Optical Denature Cycling, you **must** make sure that your initial hold is labelled as a calibration step. Otherwise, the Cycling step will include the calibration after the initial hold, causing a cool to the lower temperature in the calibration melt. The profile preview will indicate this situation :



To correct this problem, tick the **Calibration Step** box in the initial hold step.

Gain Calibration

When setting up a new run with reactions that have not previously been run on the Rotor-Gene it is helpful to use the Gain Adjustment function. This screen allows you to set the Gains of each of the channels and the set temperature.

Note: The gain adjustment will never be 100% correct. This can be due to changes in fluorescence after the first hold step. Nevertheless, the result of the gain adjustment will give you a good indication on what fluorescence level the run will be started.

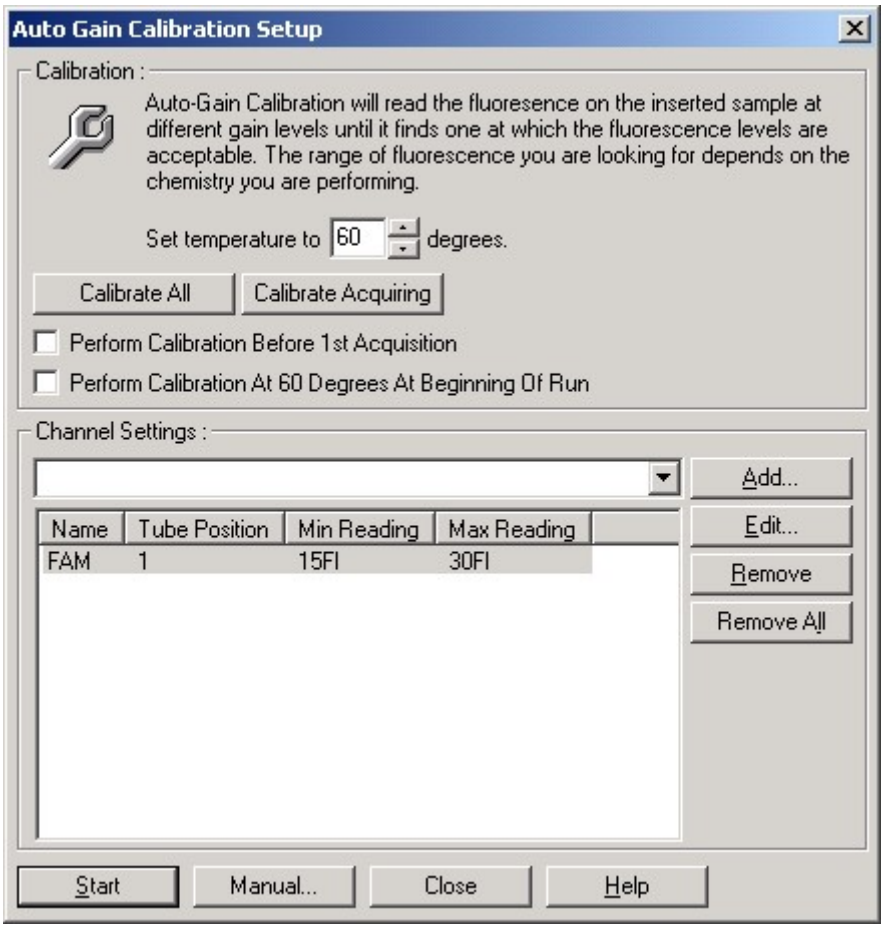
The Gain for each channel is -10 to 10, -10 = least sensitive and 10 = most sensitive.

When running reactions for the first time it is advisable to prepare a test sample containing all reaction components. This is then placed in the machine and Gain Adjustment is run to determine the best Gain setting.

Auto-Calibration

This window lets you calibrate your machine by automatically adjusting your Gain settings until the readings for all selected channels fall below a certain threshold. You can select to calibrate all channels, or just those that you will be using in the current run.

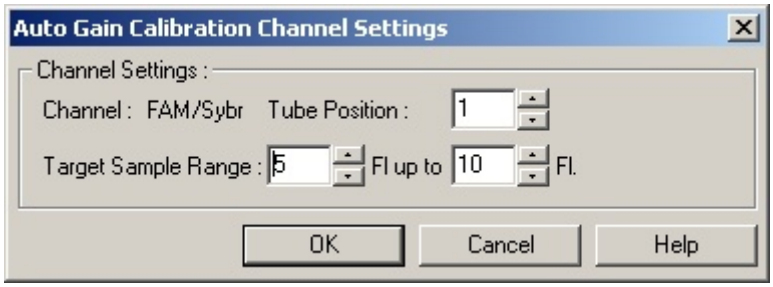
Set temperature to ... Before reading, the machine will be heated or cooled to match the given temperature.



Calibrate All / Calibrate Acquiring: "Calibrate All" will attempt to calibrate for all channels known by the software. Selecting "Calibrate Acquiring" will instead only calibrate those that you have used in the thermal profile defined in the run (cycling and melt).

Channel Settings: This is a pull down menu allowing you to add additional channels to the gain adjustment window. Choose the channel of interest and **Add**.

Edit... Opens a window where the fluorescence range of the sample can be determined. The auto-calibration process begins on Gain 5, reading from each channel. It chooses the first Gain, which has a fluorescence reading equal to or below the level you set in this box. In the example below, tube position 1 was chosen with a target sample range between 5 and 10 Fl.



Remove and Remove all: Removes the highlighted channel or all channels.

Start: Begins the calibration process. A Gain will be chosen which is within the given range. If no value is found within the range, the closest match will be chosen.

Manual: Opens the Manual Gain Adjustment screen (see below).

Perform Adjustment Before 1st Acquisition: This tick box performs the Gain Adjustment at the first cycle where data acquisition occurs. This is useful as with some probes after the initial hold the background fluorescence of the probes can change substantially. This is generally the recommended auto-gain option.

Perform Adjustment At [x] Degrees At Beginning of Run: This tick box performs the Gain Adjustment just before starting the run. The machine is heated to the given temperature, the gain adjustment is performed, and then cycling begins on the first step, usually a Denature. This can be useful when a calibration during the run may impact too much on the time spent on the initial step. Usually, however, **Perform Adjustment Before 1st Acquisition** is preferred as calibration is performed as close as possible to run conditions.

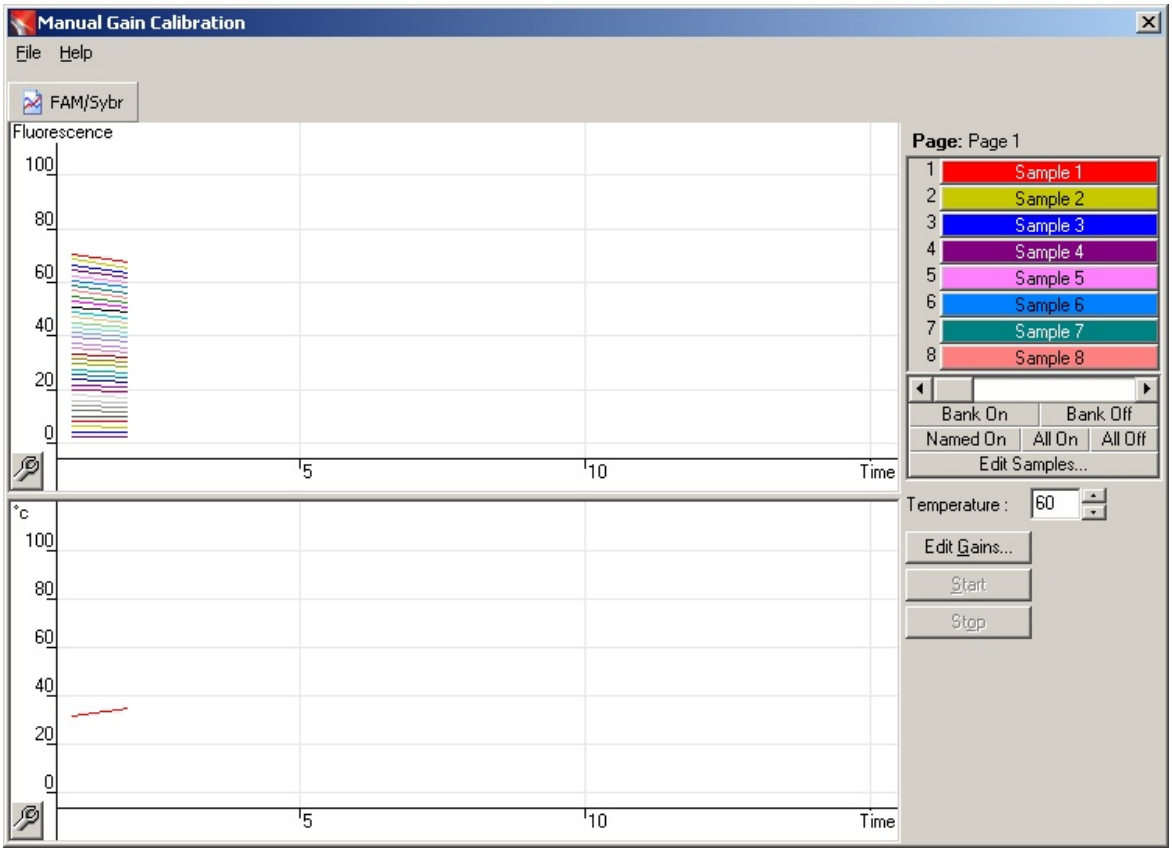
Changing Gain During a Run: If the Gain at the beginning of the run was accidentally chosen to high or to low it can be changed within the first ten cycles. A vertical line in the main screen will appear where the Gain has been changed. Due to the drop or increase in fluorescence the cycles before the change will be excluded from the analysis.

A good rule of thumb is to have fluorescence settings for:

- SYBR Green between 2 and 3
- Probe runs between 5 and 10
- Quenched FRET runs between 60 and 70.

Manual Calibration

This window lets you view in real time the fluorescent readings at any given temperature. It is used when the background of a sample is unknown and therefore the gain must be determined to ensure the sample signal is sufficient to detect.



By default, all samples are toggled on. The display samples can be toggled using the right-hand selector as in the main experiment workspace.

It takes approximately 4 seconds to acquire data per channel. During this time the user interface is deactivated and so it is best to 1) Start the test 2) wait for the temperature to stabilize 3) note the end point fluorescence (Fl) reading 4) stop the unit 5) make the appropriate Gain change and 6) restart the unit.

Temperature: Change this value to set the temperature of the machine. **Note: The temperature is not adjusted while the machine is operating. You should restart the machine to apply changes made to the temperature.** Adjust the temperature on the Gain Adjustment screen to reflect the required acquisition temperature for the run.

Edit Gains: Opens the Edit Gains window. The Gains will be sent the next time the machine is started in the calibrate window.

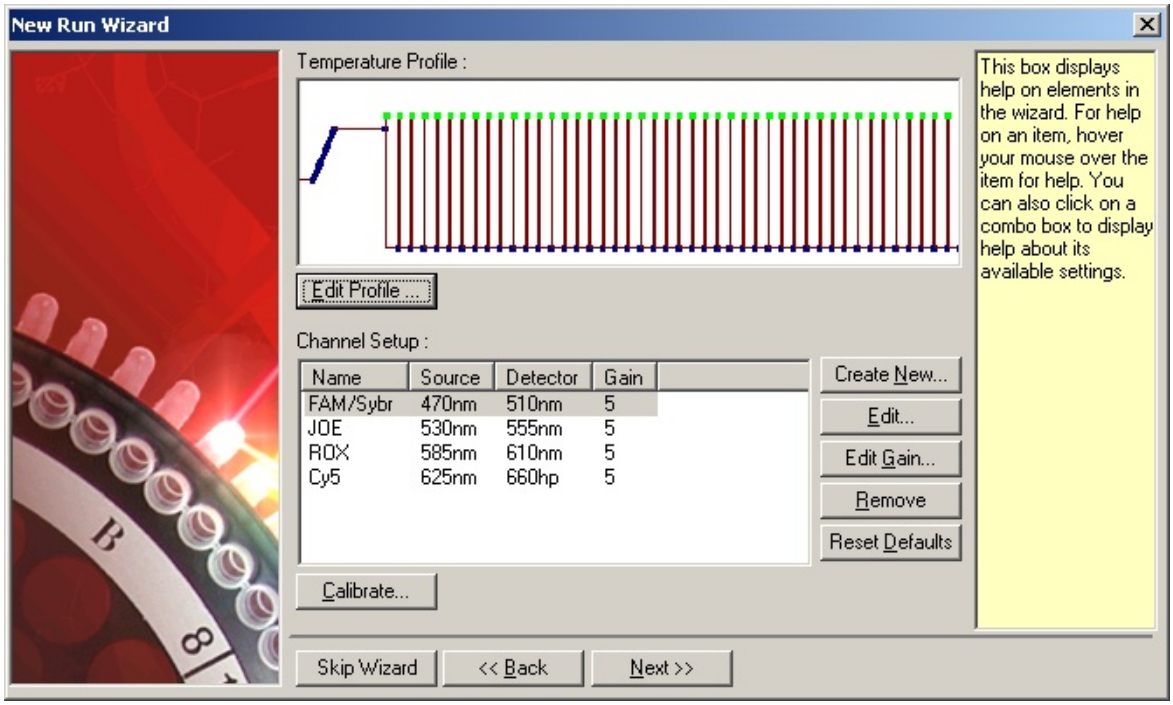
Start: Begins the run, setting the machine temperature to that displayed on the screen. The temperature and channel graphs will start to display data.

Stop: Stops the run. If the run is still acquiring data when you click the button, then the machine will first finish acquiring, and then stop the machine. This process can take up to 5 seconds for each acquiring channel.

NOTE: The aim of the Gain setting is to have all data on the screen. The Gain doesn't influence your data. If the Gain was chosen too high, curves might go off scale and data

could be lost. It is therefore a good idea to start a SYBR-Green I or dual-labeled probe run with a low fluorescence close to zero (an increase is expected) and a quenched FRET run with a higher fluorescence, close to 100 units (a decrease is expected).

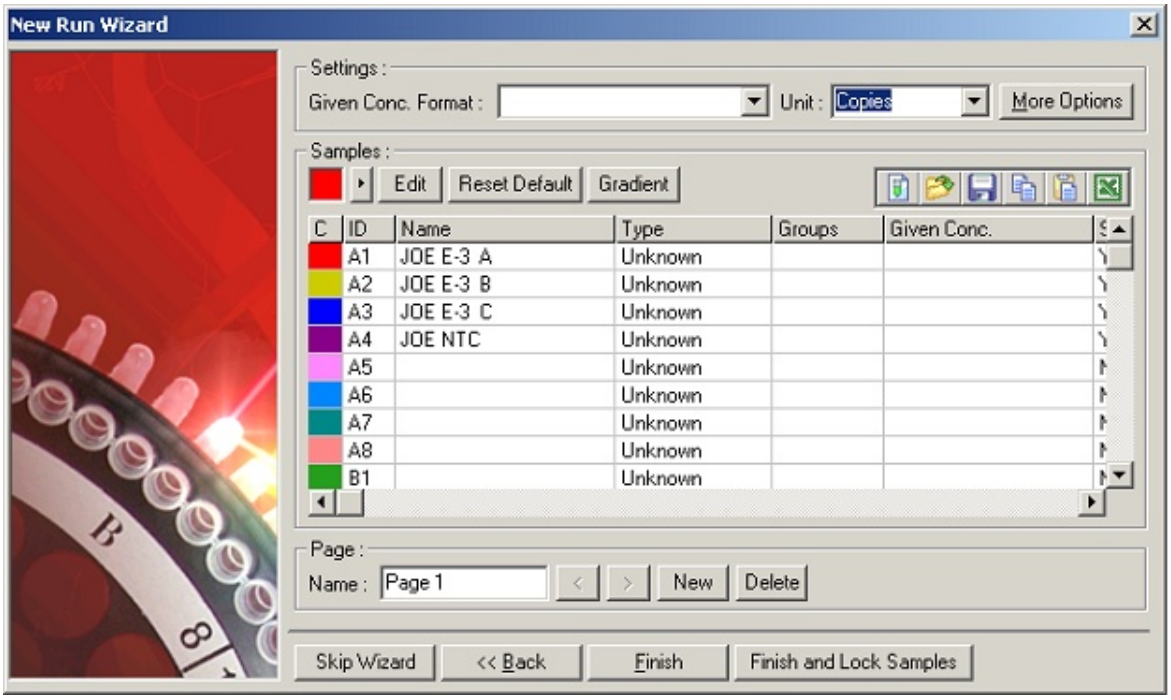
5.2.4 Page 3



The last screen gives a summary of what you are about to run. Always check the parameters. If you are satisfied with the parameters click **Start Run** and you will be prompted for a file name.

5.2.5 Page 4

Once the run has begun, you can enter in sample types and descriptions while you wait for it to complete. The functionality of this screen is identical to the Sample Editor. You may also elect to complete sample information after the run has finished.



The Finish and Lock Samples button is optional. It allows you to close the screen and prevent the sample names from being modified. More information about this, and other security features is available in Security Menu.

6 Functional Overview

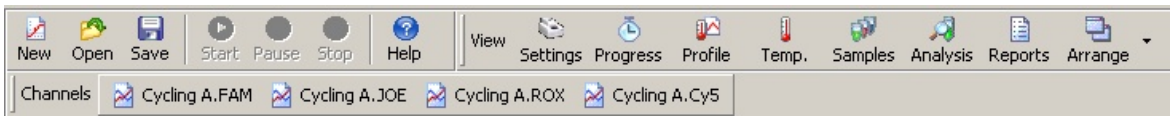
The following chapter will help to familiarize you with elements in the Rotor-Gene user interface.

6.1 Workspace

The Rotor-Gene workspace is the backdrop of the main window. This is the area in which you can open up graphs of raw data, temperature and analysis results. If you have several windows opened concurrently, you can organize them by clicking the **Arrange** button on the toolbar. There are several options available that you can access by clicking on the **Down Arrow** next to that button.

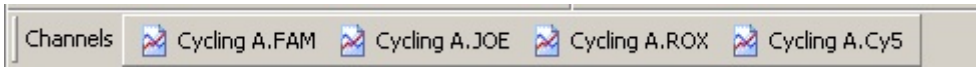
6.2 Toolbar Workspace

These buttons are shortcuts to frequently used operations. These commands can also be accessed via their corresponding menu items of the same name.

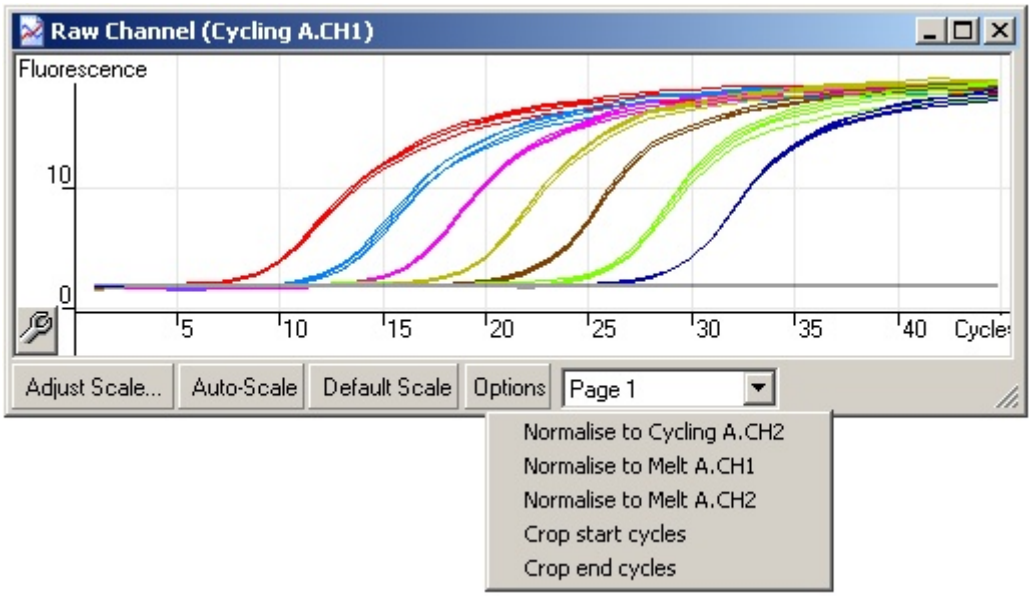


6.3 View Raw Channels

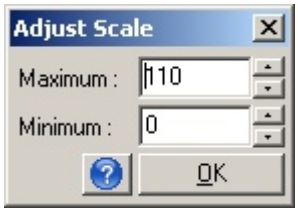
Click on these buttons to view the raw (non-analysed) data from particular channels in the run.



When viewing channel data, a number of options are available to change the presentation of the data. The channels may also be transformed to facilitate more unusual forms of analysis.



Adjust Scale will bring up a window in which you can manually enter a scale, or interactively select one. To chose this option simply press the right mouse button over the appropriate screen.



Auto-Scale attempts to fit the scale to the maximum and minimum readings in the data.

Default will reset the scale to display from 0-100 fluorescence units.

Spanner icon: See the section Spanner Icon for more information on the options provided by this button.

Options: Displays the drop-down menu indicated above, providing a number of options for transformation of the raw amplification plot.

Normalise to ...: Creates a new channel which contains the data after normalisation with the selected channel. This is useful for normalisation with a passive reference such as ROX. While the Rotor-Gene does not require normalisation to a passive reference, this is provided to preserve compatibility with assays operating on other instruments.

Crop start cycles: Creates a new channel in which some start cycles have been removed. This is useful if large jumps are observed in the initial cycles, as can occur when using certain chemistries.

Crop end cycles: Creates a new channel in which some end cycles have been removed.

Page 1: Indicates the currently selected **Sample Page** used to display the raw data plots. The Sample Editor allows for the creation of multiple sample definitions, allowing data to be viewed with varying line thickness, sample definitions and other display options. This is of particular use if Relative Quantitation is being performed in a single channel, as the operator can easily toggle the view between the Gene of Interest and Housekeeper samples through the definition of two sample pages.

6.4 Toggling Samples

At the right-hand side of the screen is a combined sample legend and toggler. Use this control to configure which samples are included in the display and results. Samples with a cell background in vivid colour are displayed while semi-greyed samples are not. The **Scroll Bar** is used to display the next group of samples. You can toggle samples individually by clicking on them, or you can hide/show all samples currently visible in the list (the current 'bank') by clicking on **Bank on/ Bank off** buttons.

Note: The number of displayed samples in a bank is dynamic, and depends on the screen space available.

To select a range of samples, click on a sample and drag the mouse to another sample. When you release the mouse button, the selected samples will either be toggled on or off. Clicking **Named On** will only show those samples you have given a name to; a quick way to show only relevant samples. Clicking **All On /All Off** will display all or none of the samples in the rotor respectively. Pressing the **Edit Samples…** button opens the sample editor window where sample names, types and standards concentrations can be edited (see Edit Samples).

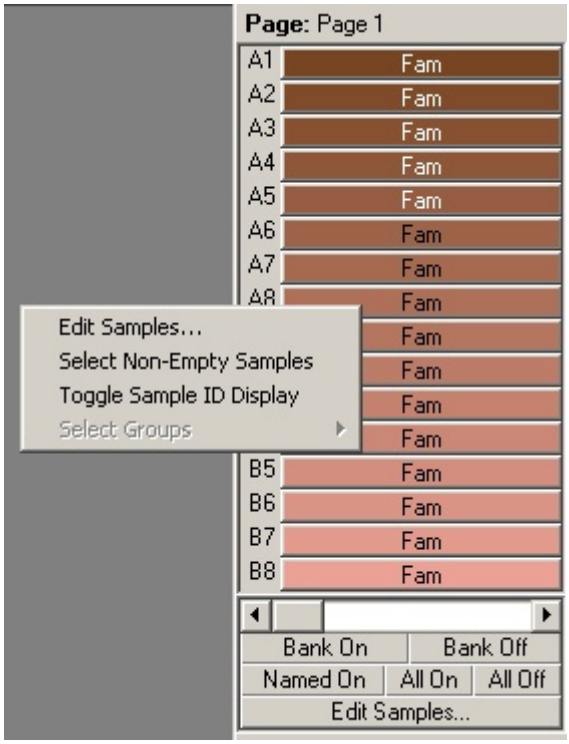
Page: This label at the top of the selector indicates the sample page that you are viewing. You can define different sample definitions for the same run to allow varied independent analyses of the same channel. For example, you can run two standard curves in the FAM/SYBR channel and generate independent reports. More information on setting up sample pages is available in the Edit Samples section of the manual.

Toggle samples ID display: If a 72-well rotor is used the samples are shown in the format A1 to A8, B1 to B8, etc. Using the toggle samples ID display button lets the user switch to a numerical order of samples (1 to 72).

Select Non-Empty Samples: Turns off any samples that have a sample type selected as "None" in the sample editor. This ensures that only samples relevant for the analysis of the particular page are displayed.

Select Groups: If you have defined sample groups, this feature will toggle the display of these samples for the active page. Groups are arbitrary collections of samples that allow advanced reporting of statistical results. You can, for example, define groups of Treated and Untreated patient samples. Groups can be set up in the Edit Samples window.

The sample toggler, shown below. The displayed additional options are accessible by clicking with the right-mouse button:



6.5 File Menu

6.5.1 New

This screen presents you with a selection of available run templates. The New Run setup wizard guides you through the run setup. The wizard is separated into two sections.

The user can set up standard templates by placing them in the template directory (usually C:\Program Files\Rotor-Gene\Templates). These templates will then appear in this window. It is even possible to create subfolders in the templates folder to group related templates. This allows you to organise your templates if many users are using the same machine, for example.

Quick Start

Provides **SYBR® Green I**, **Dual Labelled Probe** and **Quenched FRET** templates with default temperature cycling and acquisition parameters that are common for these detection chemistries. These profiles can be altered during the wizard setup.

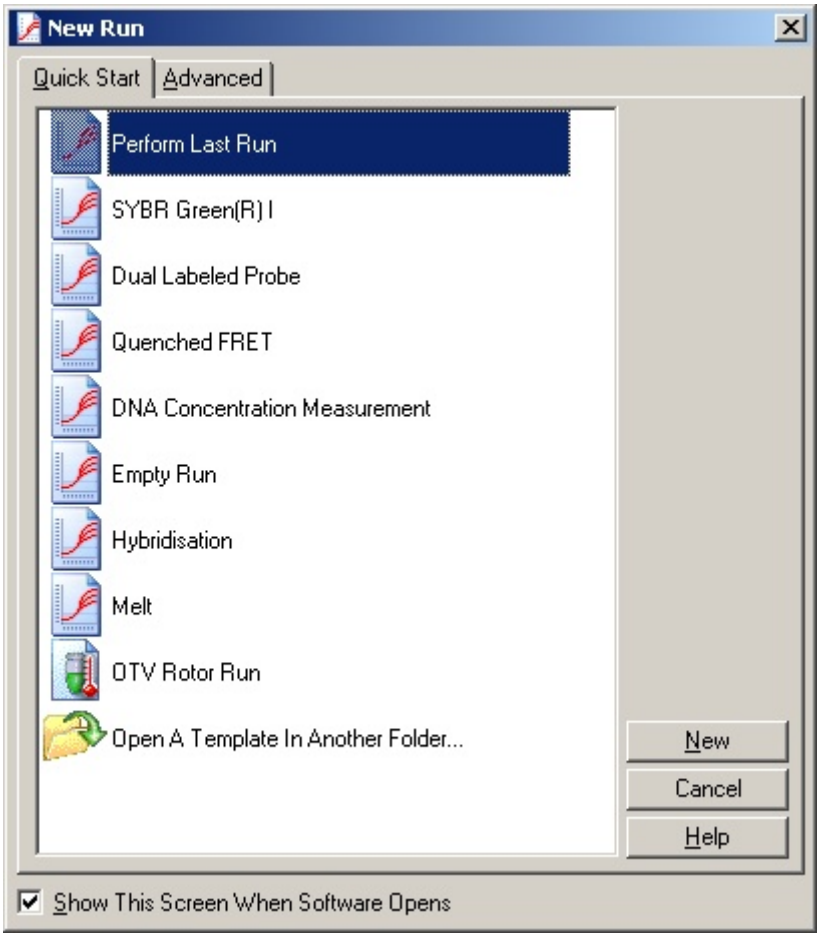
Perform Last Run template defaults all settings for the run to that of the last run that was opened in the software.

Advanced

The Advanced wizard allows the user options to alter more parameters than the Quick Start wizard.

Perform Last Run imports the initial settings from the last run opened in the software.

Open A Template In Another Folder... enables user to browse to a template saved in a different folder.



New Run

New...: Initiates the wizard setup of the run using the selected template's configuration.

Cancel: Closes this window.

Help: Opens on-line help.

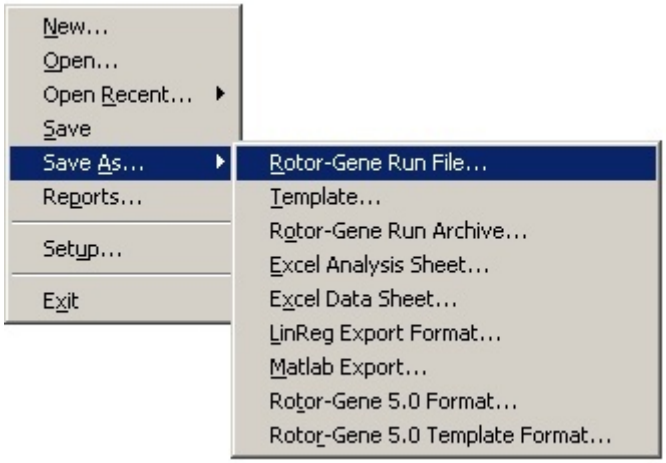
Show this screen when software opens: If this option is ticked the wizard is displayed when the software is initiated.

6.5.2 Opening and Saving

Open...: Opens a previously saved Rotor-Gene Run File (.rex) or Rotor-Gene Run Archive (.rea file).

Open Recent...: Displays the last four files that have been opened or saved.

Save: Saves any changes that have been made to a run file.



Save As...: Use this function to save the run file or data in various formats. The options are as follows:

Rotor-Gene Run File...: Saves a copy of the file. The user can change the name and the save location of the run. This is the default format.

Template...: Saves the profile setup and associated settings but not the run data. The template can be used to initiate future runs.

Rotor-Gene Run Archive...: Saves in a more compact file format. You should save files in this format before they are emailed. This both reduces the time required to send the file, and ensures that files are not corrupted by mail clients.

Excel Analysis Sheet...: Exports all the analysis in the current run into a single data sheet.

Excel Data Sheet...: Exports all of the raw channels to an excel sheet. Only the samples you have selected will be exported.

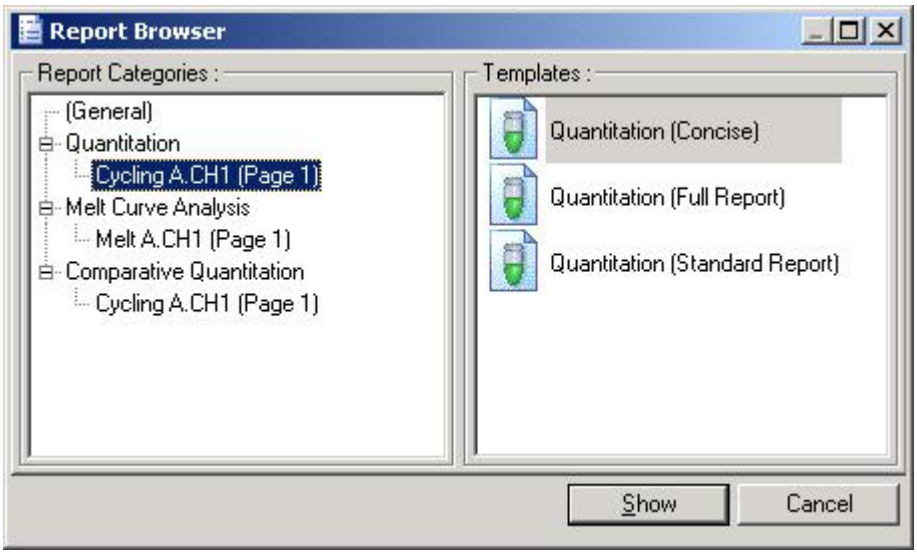
Matlab Export...: Exports the data into a format which can be read by the scientific package Matlab (or its Open-Source equivalent, Octave). This may be useful for those involved in methods research.

Rotor-Gene 5.0 Format...: The run can be saved as a version 5.0 of the Rotor-Gene software so that the run can be viewed in that version of the software. Some data such as sample pages cannot be saved in this format, and so is lost.

Rotor-Gene 5.0 Template Format...: The run can be saved in a version 5.0 template format.

6.5.3 Report

The Report menu brings up the **Report Browser** window. If the data has already been analysed, it is possible to display the report of that analysis directly through the Report Browser rather than through the particular analysis module. A number of report templates are offered with varying degrees of detail.



6.5.4 Setup

Rotor-Gene Setup should be completed during the installation of the instrument. Using the **Setup** function you can change the settings of the instrument. See the section Installation for more information.

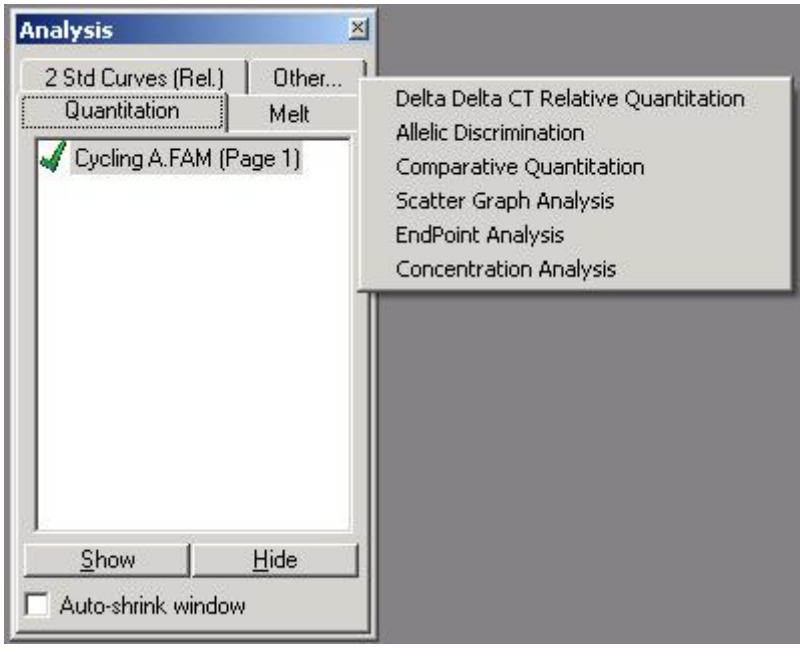
6.6 Analysis Menu

6.6.1 Analysis Toolbar

Clicking the **Analysis** menu brings up the **Analysis** floating window.

This window allow you to create new analyses and displays existing ones. The tabs enable you to select a method of analysis to perform. Once you have done this, a list of the channels which can be analysed using this method will be listed below. Multiple assays run in the same channel can be analysed independently, provided they have been set up as separate pages in the sample editor. Pages that have already been analysed will have a green tick next to them, e.g. the FAM channel (Page 1) shown in the screen shot. This means that threshold and normalization settings have been remembered for this analysis. To analyze a channel, or view an existing channel,

simply double-click on the channel to view. The specific analysis window will then appear.



Auto-shrink Window: Ticking the box shrinks the window when it is not used. Moving the cursor over the window enlarges the window again.

Organizing Your Workspace: Each time you double-click on a new analysis, its windows will be arranged to fit in with those already on the screen. With many windows, this can be cumbersome. Simply close the windows you do not require, then click **Arrange** on the toolbar. The windows will automatically be rearranged according to the **Smart Tiling** method. You can select another method of arrangement by clicking the **down arrow** next to the toolbar button.

Clicking the right mouse button over the analysis window also allows to **Show, Hide** or **Remove Analysis**.

6.6.2 Quantitation

Double click quantitation or press **Show** to open the channel of interest. Three windows will be opened automatically, the main screen, the standard curve and the results. Going from left to right, the following buttons are displayed in the main window:

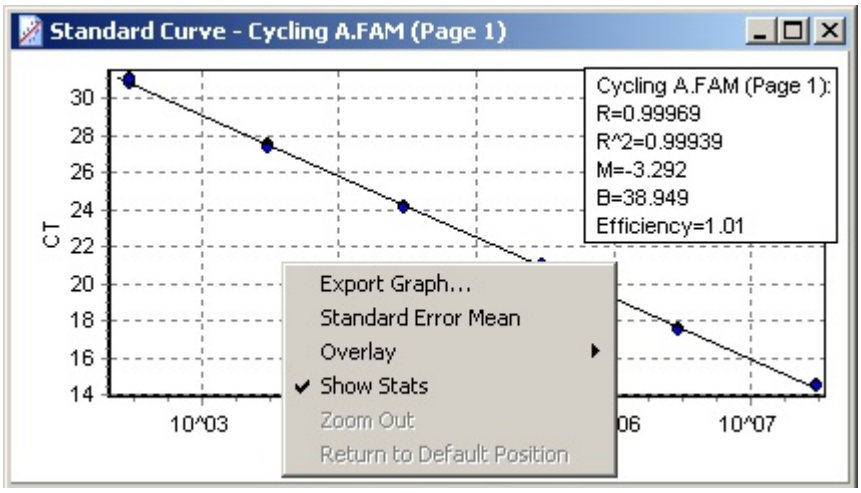
Reports

Reports: Opens the Quantitation Report selection window where you can choose a report to preview of the currently selected Quantitation analysis. There are three different options, Standard Report, Full Report and Concise Report.

Using the buttons on the top, the reports can be **printed, saved, emailed** or **exported to Word**.

Standard Curve

Std. Curve: This button opens the standard curve graph. By default, this window is opened when an analysis is opened. If you close the window, it can be re-opened by using this command.



On the standard curve the values are recalculated dynamically as the threshold level is varied, by clicking and dragging.

Blue dots on the curve show the samples that have been defined as standards and **red dots** show the unknown sample data points.

NOTE: If redefining standards to recalculate the standard curve, toggling the standard colors ON or OFF will remove it from standard curve calculation. Removing standards from your graph to increase the R^2 value is not scientifically valid. A failed standard is an indication that your samples may have also failed, and so must be included in your results.

Efficiency: The Reaction Efficiency of the run. This value is discussed in more detail in Slope, Amplification, Reaction Efficiency**.**

R^2-value (correlation coefficient): The R^2 value, or R^2 value (as displayed with the superscript), is the percentage of the data which is consistent with the statistical hypothesis. In the

Quantitation context, this is the percentage of the data which matches the hypothesis that the given standards form a standard curve. If the R^2 value is low, then the given standards cannot be easily fit onto a line of best fit. This means that the results obtained (ie. the calculated concentrations) may not be reliable. A good R^2-value is around 0.99.

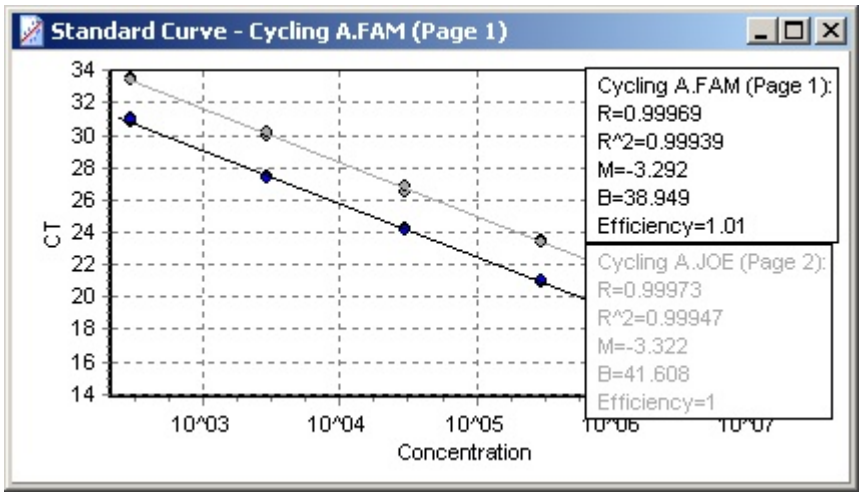
NB: It is still possible to achieve a high R^2 value with a poor standard curve, if not enough standards have been run. The R^2 value will improve as the number of standards decreases. To get a more accurate indication of the error, use the confidence intervals on the calculated concentrations as a guide of variation.

R-value (square root of correlation coefficient): The R-value of the calculation is the square root of the R^2 value. Unless you have a specific statistical application, the R^2 value is more useful in determining correlation.

According to the formula **y = mx + b** the slope (**M**) and the intercept (**B**) of a standard curve are automatically calculated and shown in the top right corner of the standard curve window.

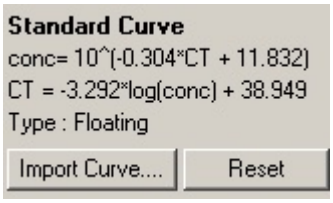
Export to JPEG...: With the pointer on the standard curve, click on the right button to show the option to export as a JPEG.

Overlay: When multiple quantitation runs have been performed in the same run, it is possible to overlay the standard curves in the same window. This is useful for graphically viewing the difference between different thresholds on the statistical results. Below is a screenshot of this feature:



Standard Curve Calculation

"conc=...*C_T + ..." and **"C_T=..."** represent two rearrangements of the equation used to relate CT values and concentrations. If you are referring to publications, the "**C_T=...**" formula will be most of use, as it in the same format. Type can be either floating or fixed. If floating, an optimal standard curve equation is calculated as you move the threshold. If Fixed, the equation does not change because it has been imported from another run.



Import Standard Curve

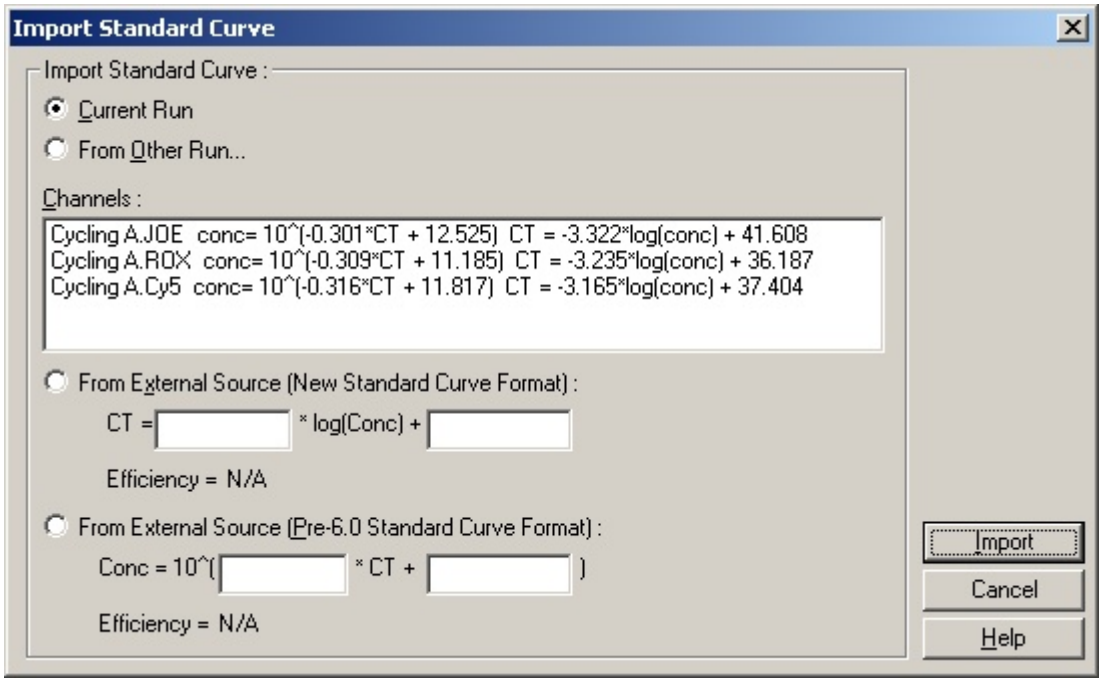
Importing a standard curve allows you to perform estimates of concentrations when a standard curve is not available in a run and you are certain that the reaction efficiency has not varied between the two runs. You can import curves from another channel, or from another run by clicking on Import Curve.

You can choose to **Adjust** the standard curve, or not to adjust. Adjusting means that only the efficiency of the source standard curve is imported into the current run. Whether you should adjust the standard curve or not depends on the chemistry application.

To adjust a standard curve, you use a reference in the target run with a known concentration. You can define a reference by setting a sample's type to Standard, and entering a concentration value in the Sample Editor. Multiple copies of the same reference can be entered to improve accuracy of the method. Note that you cannot define more than 1 reference concentration. For example, it is possible to have 3 replicate references of 1000 copies, but not to have one reference of 1000 copies, and another with 100 copies in the same run.

Once the curve has been imported, the standard curve type will be changed to Fixed. Click **Reset** to set the curve type back to Floating.

Below is a screenshot of the Import Standard Curve screen:



Using this screen, you can import the standard curve from another channel you have analysed in the current run, or you can load a standard curve from another run.

Current Run: When this option button is selected, quantitation analyses on other channels from this run will be listed with their corresponding standard curves.

From Other Run: Selecting this option button will bring up an Open Dialog in which you can select a run file to open. If any quantitation analysis has been performed for the run, you will see standard curves listed for each channel analysed.

Channels List: Lists the analysed channels and their respective standard curve formulas.

From External Source: You can now also type in M and B values directly. This is useful in cases where the values are from an external source, such as an Excel spreadsheet.

Invert Raw Data

Some chemistries produce a fluorescent readout that decreases exponentially instead of increasing. It is still possible to analyse these using Quantitation, but the "Invert Raw Data" checkbox should be ticked.

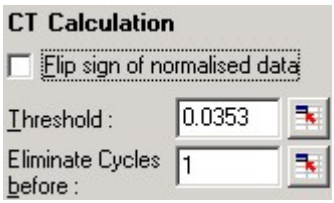
For all other quantitation analysis, this option must remain unticked.

NB: Techniques such as Quenched FRET or the use of Bodipy(R) which use decreasing signals to quantitate may have less accurate results than those that increase. Such techniques have not been widely verified yet in the scientific community.

Calculation of CT

C_T-Calculation: The C_T value is the value where the amplification curve crosses the threshold line. By setting a threshold line and calculating the intersection with each of the sample curves, the C_T values for each sample are established.

Threshold: (Manual Set): To set the threshold click on the icon (grid with red arrow) then click and hold on the graph and drag a threshold line to the desired level, or enter a log value. Alternatively the Auto-Find Threshold function can be used to automatically determine the best level. When setting a threshold manually, it should be set in the exponential phase of the run, significantly above the background level to avoid noise.



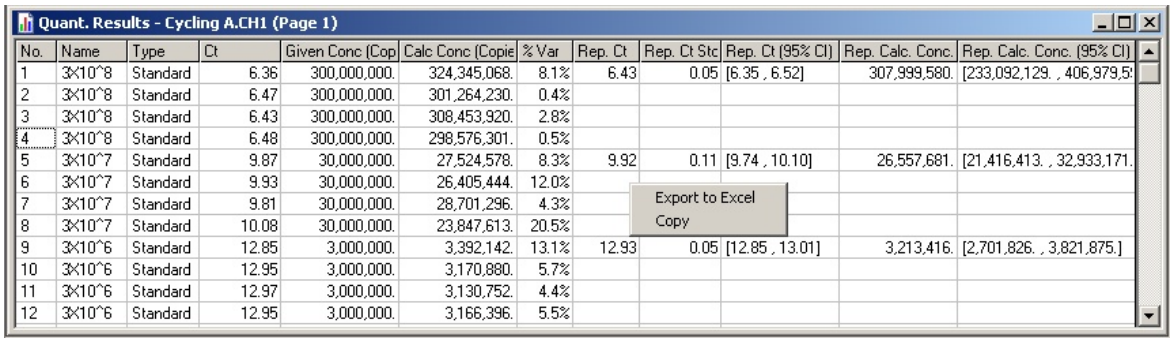
Eliminate Cycles before: To set, click on the icon (grid with red arrow) then click and hold on the graph and drag the threshold line to the right. This eliminates the threshold line for low cycle numbers.

Note: This is useful when there is noise on the signal during the initial cycles.

Auto Find Threshold: The automatic threshold function will scan the darkened region of the graph to find a threshold setting which delivers optimal estimates of given concentrations. You can change the region to be modified by entering new upper and lower bounds in the text boxes. For most quantitation analyses, the default region is suitable. Based on the standards that have been defined the function then scans the range of threshold levels to obtain the best fit of the standard curve through the samples that have been defined as standards, (i.e. maximizes the R value to approach 1.0).

Results

Opens the quantitation results grid. By default, this window is opened when you open an analysis. If you close it, it can be re-opened by using this command.



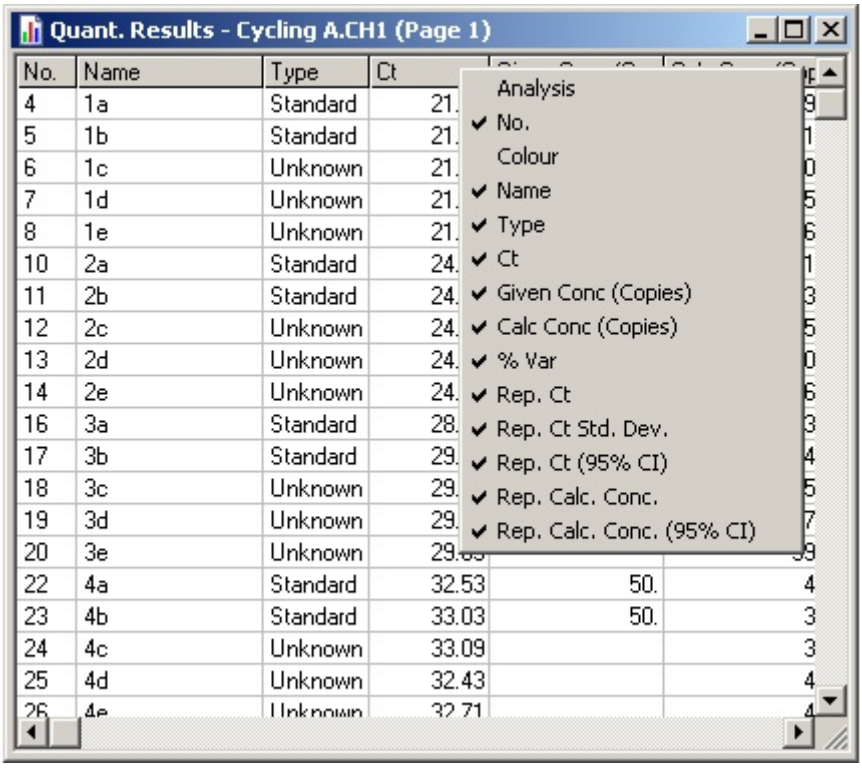
No.	Name	Type	Ct	Given Conc (Cop	Calc Conc (Copie	% Var	Rep. Ct	Rep. Ct Stc	Rep. Ct (95% CI)	Rep. Calc. Conc.	Rep. Calc. Conc. (95% CI)
1	3X10^8	Standard	6.36	300,000,000.	324,345,068.	8.1%	6.43	0.05	[6.35 , 6.52]	307,999,580.	[233,092,129. , 406,979,5
2	3X10^8	Standard	6.47	300,000,000.	301,264,230.	0.4%					
3	3X10^8	Standard	6.43	300,000,000.	308,453,920.	2.8%					
4	3X10^8	Standard	6.48	300,000,000.	298,576,301.	0.5%					
5	3X10^7	Standard	9.87	30,000,000.	27,524,578.	8.3%	9.92	0.11	[9.74 , 10.10]	26,557,681.	[21,416,413. , 32,933,171.
6	3X10^7	Standard	9.93	30,000,000.	26,405,444.	12.0%					
7	3X10^7	Standard	9.81	30,000,000.	28,701,296.	4.3%					
8	3X10^7	Standard	10.08	30,000,000.	23,847,613.	20.5%					
9	3X10^6	Standard	12.85	3,000,000.	3,392,142.	13.1%	12.93	0.05	[12.85 , 13.01]	3,213,416.	[2,701,826. , 3,821,875.]
10	3X10^6	Standard	12.95	3,000,000.	3,170,880.	5.7%					
11	3X10^6	Standard	12.97	3,000,000.	3,130,752.	4.4%					
12	3X10^6	Standard	12.95	3,000,000.	3,166,396.	5.5%					

The results obtained from the run are summarized in a table. Clicking the right mouse button, and selecting **Export to Excel** will export the table to Excel. There is no need to open the Excel program, as this will be done automatically. If you would like to copy the data into an existing spreadsheet, choose the **Copy** option instead, then open your spreadsheet, then select paste.

Explanation of Results:

%Var	The percentage variation between the calculated and the given concentration. %Var=*Abs(Calculated/Given-1)*
Rep. C_T	The average C_T of all samples with the same name as this sample.
Rep. C_T Std. Dev.	The standard deviation of the C_T value of all samples with the same name as this sample.
Rep. C_T 95% C.I.	A C_T range which, statistically, accounts for 95% of the variation in the C_T value. This is a conservative statistical measure which can be used as a quality measure. This window can be tightened by running more replicates, or by having less variation in the replicates.
Rep. Calc. Conc	The calculated concentration for all the samples with the same name. Note: This is not the simple average of the calculated concentrations, but rather the "Geometric Mean", which is a mathematically more suitable average.
Rep. Calc. Conc 95% C.I.	A range of concentrations which accounts for 95% of the variation in the individual sample as well as the linear regression model on which it is based. An interpretation of this measure is "the range of concentrations that could be expected 95% of the time if this run was performed repeatedly with the same amount of variation". This is a conservative estimate, and the range can be quite large due to the variation inherent in any real-time analysis. This range can be large if standards are run with different concentrations to the unknowns, if a small number of replicates are used, or if there is significant variation. **IMPORTANT: The variation which are reported by this measure are not due to the Rotor-Gene machine, but inherent in the exponential process of real-time amplification. Similar tests performed on competitor machines would yield still greater variation due to the lower temperature uniformity of block systems. As a cross-machine comparison, use the C_T standard deviation, as this should be supported by all major vendors.**

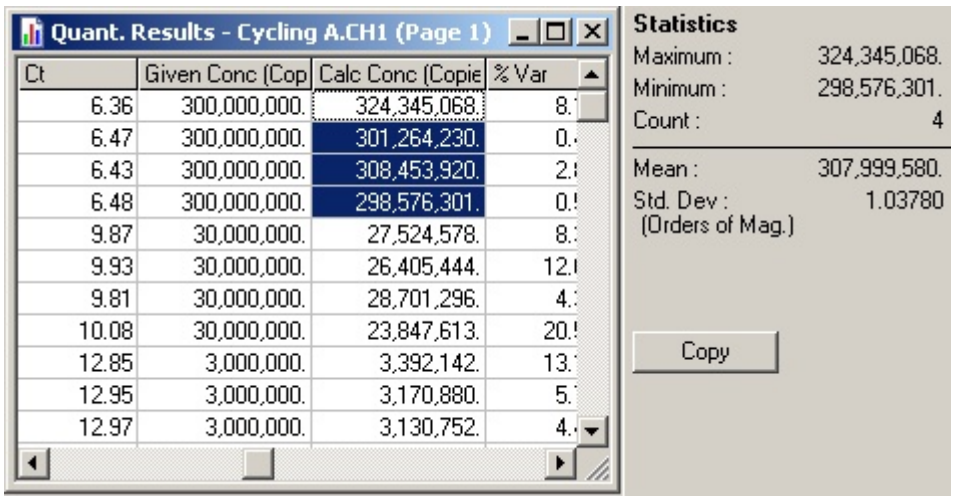
TIP: You can toggle each of the columns on or off by right-clicking on the column:



More detailed information on the confidence intervals is available in the appendix. Our thanks goes to Peter Cook from the Mathematics Department of the University of NSW, Sydney, Australia, whose help was invaluable in verifying the mathematical approaches used.

To make calculations easier, a feature called **AutoStat** is introduced which automatically calculates the Average, Standard deviation, Minimum and Maximum values of samples of interest. Simply select the results of interest by dragging with the left mouse button, and the aggregate values are given in a small table displayed below the sample list on the right-hand side of the screen.

In this screenshot, the concentrations of several samples are analysed:



Important: The AutoStat feature is context-aware. This means that it will, where possible, only generate information which is useful. For example:

- It is not possible to obtain a 95% Confidence Interval from a set of selected calculated concentrations because the regression model must be taken into account as well.
- The "Orders of Magnitude" standard deviation is reported for Calculated Concentrations rather than an absolute value. This value can be understood as a percentage variation. For example, a value of 1.03780 represents a 3.78% variation (289,073 -- 311,340)=(300,000/1.0378 -- 300,000*1.0378). Reporting an absolute error does not make sense at all for a standard curve. We could report the error at the lowest concentration to create a perceived low error (+/- 3 copies) or report the error at the high end (+/- 3,000,000 copies). For this reason, only a valid measurement, the orders of magnitude, is reported.
- For Calculated Concentrations, the Geometric Mean is used instead of the Arithmetic Mean. This is needed to account for the exponential nature of Real-Time Amplification. To demonstrate this, imagine that a 2-fold dilution with 1, 2, 8 and 16 copies was taken. If we take the average, we expect to get 4 copies, because it is the "middle" of the dilution series. If we take the simple average, we obtain 6.75, which is unexpected. If we use the Geometric Mean, we get (1*2*8*16)^(1/4) = 4 copies as we should intuitively. More information on Geometric means can be found at http://mathworld.wolfram.com/GeometricMean.html.

Dynamic Tube Normalisation

This option is ticked by default and is used to determine the average background of each individual sample just before amplification commences. Standard Normalization simply takes the first five cycles and uses this as an indicator for the 'background' level of each sample. All data points for the sample are then divided by this value to normalize the data. This process is then repeated for all samples. This can be inaccurate as for some samples the background level over the first five cycles may not be indicative of the background level just prior to amplification. Dynamic Tube Normalization uses the second derivative of each sample trace to determine a starting point for each sample. The background level is then averaged from cycle 1 up to this starting cycle number for each sample. **This method gives the most precise quantitation results.** Alternatively with some data sets it may be necessary to disable the dynamic tube normalization. If this is the case the average background for each of the samples is only calculated over the first 5 cycles. If the background is not constant over the cycles before amplification it will result in less precise data.

Noise Slope Correction

The background fluorescence (Fl) of a sample should ideally remain constant before amplification. However, sometimes the Fl-level can show an increase or decrease due to the effect of the chemistry being run and produce a skewed noise level. The Noise Slope Correction option uses a line-of-best-fit to determine the noise level instead of an average, and normalizes to that instead. Turning on this option can tighten replicates if your sample baselines are noticeably sloped.
This function improves the data when raw data backgrounds are seen to slope upward or downward before the amplification Takeoff point (C_T). It is very helpful for runs when for example the FAM background is seen to creep upwards due to gradual probe autohydrolysis.

Ignore First

The first couple of cycles in a quantitation run are not usually representative of the rest of the run. For this reason, you may get better results if you select to ignore the first few cycles. If the first cycles look similar to cycles after them, you will gain better results by disabling this function, as the normalization algorithm will have more data to work with. You can ignore up to ten cycles.

Quant. Settings

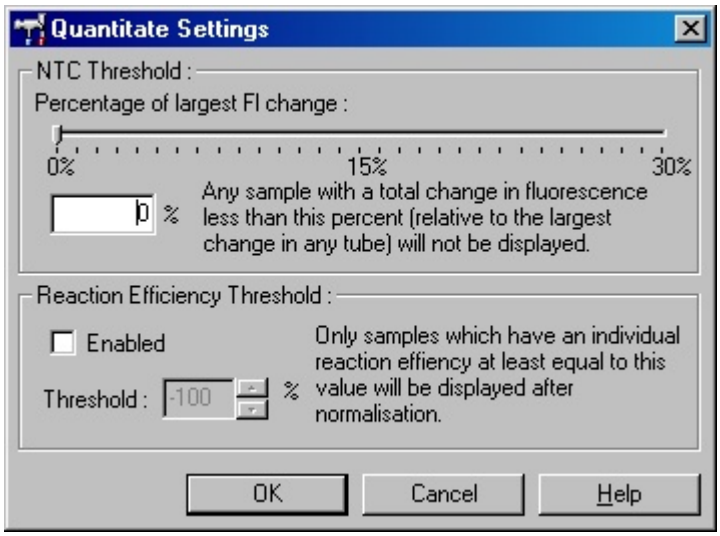
To distinguish between minor changes in fluorescence and genuine reactions in No Template Controls, two measures are provided which act through different mechanisms. The NTC Threshold is usually recommended for most applications, though you should validate the levels used in any approach.

NTC Threshold:
The quantitation setting in the quantitation screen allows excluding samples or NTCs, which have a slight drift upwards, due to probe degradation or other non PCR effects. All samples with a change below the NTC threshold will not be reported in the quantitation screen. The percentage is relative to the largest maximum change found in any tube. For example, if you have one sample which began at a background of 2Fl and went to 47Fl, then 45Fl represents 100%. An NTC threshold of 10% would consider as noise any sample with a reaction less than 4.5Fl.

Reaction Efficiency Threshold:
The Reaction Efficiency Threshold is an alternative mechanism to exclude noise samples from analysis. This normalising algorithm uses the reaction efficiency estimation techniques used in Comparative Quantitation. All samples will be excluded if they do not have a reaction efficiency of at least this level. A level of 0% indicates that, during the exponential phase, no reaction took place. 100% indicates that a completely efficient reaction took place during the exponential phase. Negative percentages indicate that during what has been guessed as the exponential phase, the fluorescent signal declined.

Current research is not conclusive on the precise levels of efficiency needed to distinguish genuine reactions from contamination and other effects. For this reason, Corbett Research recommends using this feature conservatively, with the assumption that any sample with a genuine reaction will have some visible exponential phase with some growth. Setting this value higher than 0% will exclude some samples with inefficient, but perceptible growth, whereas setting below 0% will display samples whose reading decreased during the exponential phase, and which should clearly be excluded.

NB: Comparative Quantitation requires a strong signal to work effectively. Some chemistries with low fold increases, such as LUX primers, may be inappropriate for use with this technique. You should validate your approach before applying in general.



Slope, Amplification, Reaction Efficiency

The slope (M) of a reaction (shown in the standard curve window), can be used to determine the exponential amplification and efficiency of a reaction.

The following calculations give some important results: exponential amplification = $10^{(-1/M)}$ or reaction efficiency = $[10^{(-1/M)}] - 1$. Optimal values for m, amplification and reaction efficiencies are –3.322, 2 or 1, respectively. The reaction efficiency is displayed in the quantitation report and in the standard curve window.

The slope is calculated of being the change in C_T divided by the change in log input (for example copy number). A 100% efficient amplification means a doubling of amplification product in each cycle resulting in an M value of -3.322, an amplification factor of 2 and a reaction efficiency of 1.

Given an M value of –3.322, the calculations are as follows:

Amplification value: $10^{(-1/-3.322)} = 2$,
Reaction efficiency: $[10^{(-1/-3.322)}] - 1 = 1$.

Here are two examples for two different slope values.

An M value of 3.8 means that the reaction has an amplification value of ~1.83 and a reaction efficiency of 0.83 (or 83%).
There could be several reasons for this value. If the value needs to be improved, optimization steps like primer or probe concentrations, $MgCl_2$- or SYBR-Green I concentrations could be improved.

An M value of 3 means that the reaction is more than 100% efficient. A reason for this could be an disproportionate digestion of probe compared to the amplicon produced. In addition, if the R-value is low, then statistical error can cause an unexpected reaction efficiency.

NOTE: This has changed from previous software versions. Previously, it was necessary to transform the gradient m' = -1/m. This is not necessary in this version.

Offset

Intercept:
In a formula describing the relation between two variables, the intercept is expressed with the letter "B" (Y = MX + B). The intercept is also sometimes referred to as the **Offset**.

The B value represents the C_T for a given concentration of 1 unit. By substituting 1 into the concentration formula as shown below:

$C_T = \log(1) * M + B$
$C_T = 0 * M + B$

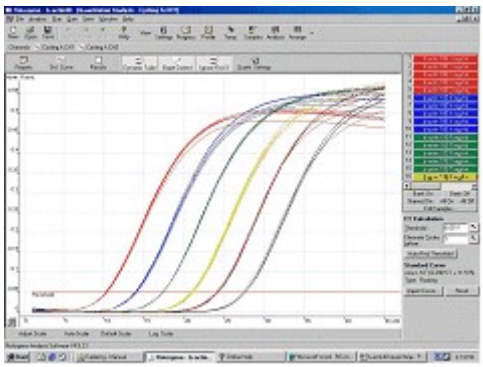
we obtain $C_T = B$ as described above.

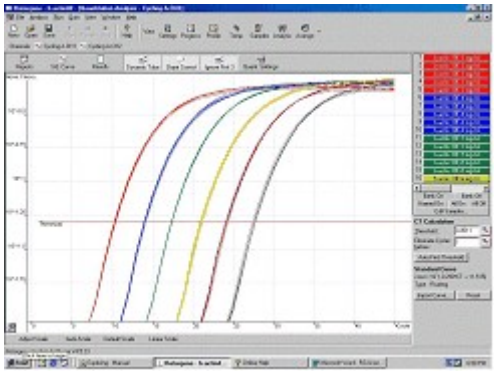
The intercept can change from run-to-run, and is less stable than the gradient. For this reason, most analysis techniques will analyse the gradient rather than the intercept.

Main Window

This screen shows that taking the average background energy for each of the samples normalizes the data, which is then displayed on a logarithmic scale.

From this graph, a threshold can be determined to calculate the C_T value for each of the samples. The C_T value denotes the cycle at which the amplification reached a critical fluorescence level. This C_T value can be related directly to the starting copy number of the sample by means of a standard curve.





Pressing **Linear Scale** on the bottom of the screen takes you directly from the **Log Scale** to the **Linear Scale** and vice versa.

Changing between these two modes only alters the display of the graphs, not the calculations. This can be verified by use of the pinpointer tool available by right-clicking on the graph. In the Log-Scale mode, smaller values are emphasised, whereas the linear scale facilitates the view of the entire reaction.

Note: This process can also be performed while the Rotor-Gene is running. This real-time monitoring of quantitation data provides the user with the possibility to gain results as soon as the curves show an exponential growth. Preliminary conclusions can be drawn and decisions made for the next run.

6.6.3 Two Standard Curve

Relative Gene Expression analysis with a Normalising Gene can be performed using the Two Standard Curve method.

The method relies on having a standard curve for each gene. The standard curves are used to quantify the concentration of each gene according to its standard curve. The expression of the Gene of Interest is then normalised with the Normalising Gene (often referred to as a Housekeeping Gene).

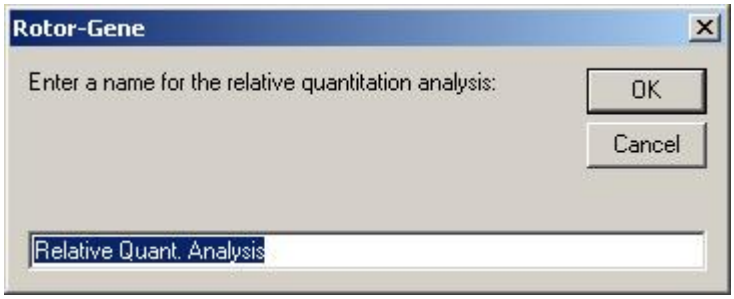
It is important that the Sample Setup is configured correctly to ensure the standards and replicate samples are designated correctly. In particular, corresponding samples must have the same name in each analysis to be correctly paired. In a multiplex reaction, where the tube positions of Gene of Interest and Housekeeper are the same, one set of sample definitions is sufficient. If performing a relative study using a single channel, two sample pages will need to be created. The first will label the tube positions with sample names for the Gene of Interest, with the other positions left unnamed. The second will label the positions used for the Housekeeper. The relative quantitation module will then match samples across the two absolute analyses based on their names.

Performing Expression Analysis using the Two Standard Curve method

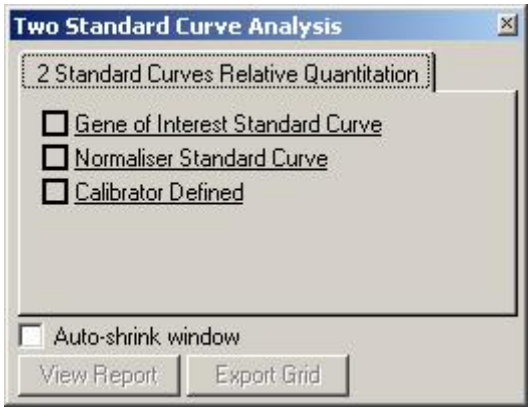
Data can firstly be analysed for each gene using absolute Quantitation Analysis. If this is not done the results for each gene will be automatically determined using the Autofind Threshold tool.

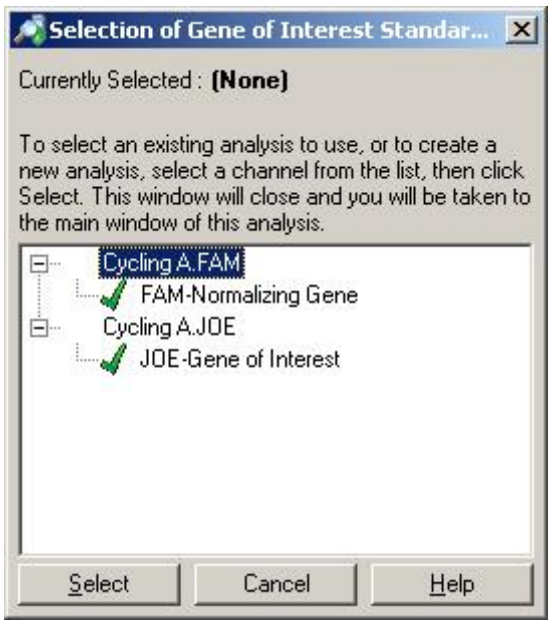
From the analysis window select the **2 Std Curve (Rel)** tab. Select **New Analysis...**

Enter a name for the analysis.

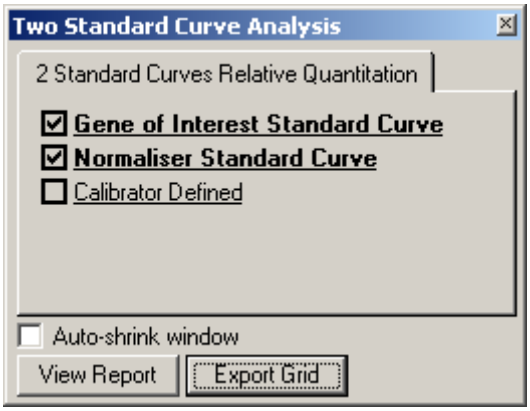


Designate the pages used for Normaliser Gene analysis and Gene of Interest analysis. For example, clicking on Gene of Interest Standard Curve will bring up the Selection of Gene of Interest Standard... window. Select the page where the Gene of Interest was Quantitated. Repeat the procedure for the Normaliser Gene. Optionally a Calibrator can be defined. If this option is selected, the calibrator is assigned a value of 1 and all other sample concentrations are calculated relative to this sample.

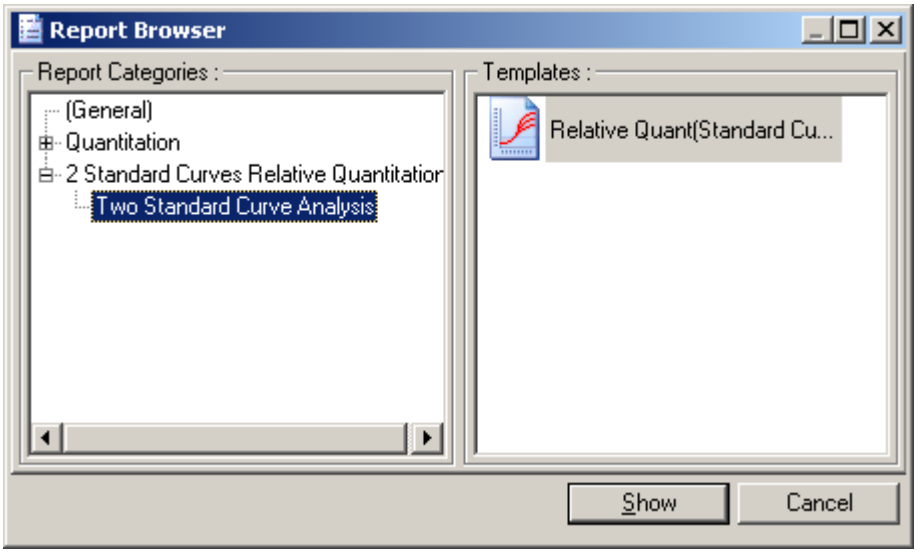




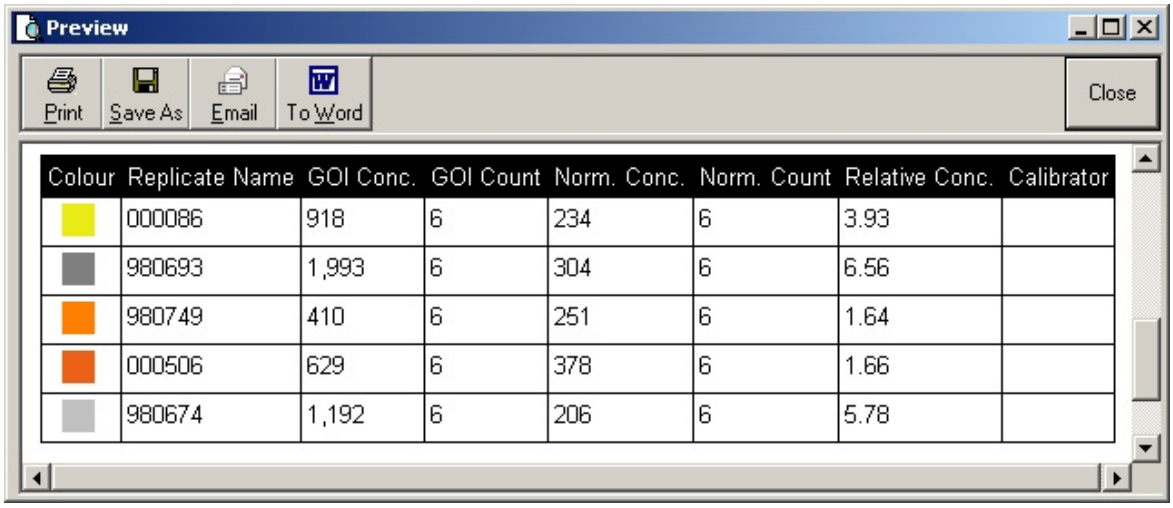
After completing the selections the options will be ticked.



Click the **View Report** button to display the Report Browser. Select the analysis with the correct name from the Report Analysis. Click the **Show** button to display the Relative Quantitation report. The Export Grid option exports the results to an excel spreadsheet. If a calibrator is selected, then the results are calculated relative to the calibrator sample, which is assigned a value of 1.



The concentrations as read from the standard curves of the unknowns for the Gene of Interest (GOI Conc.), the Normaliser Gene (Norm. Conc) as well as the relative concentration (Relative Conc.) are displayed in the results table. The results can be saved as a word file.



Colour	Replicate Name	GOI Conc.	GOI Count	Norm. Conc.	Norm. Count	Relative Conc.	Calibrator
	000086	918	6	234	6	3.93	
	980693	1,993	6	304	6	6.56	
	980749	410	6	251	6	1.64	
	000506	629	6	378	6	1.66	
	980674	1,192	6	206	6	5.78	

6.6.4 Delta Delta Ct Relative Quantitation

Comparative C_T method (delta delta C_T) is a method for Relative Gene Expression analysis.

The method implemented is descibed in the following publication:

"Analysis of relative gene expression data using real-time quantitative PCR and the 2^[-delta delta C(T)] Method."
Livak KJ & Schmittgen TD. Methods 2001 Dec;25(4): 402-408

The abstract can be found at the following link:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=11846609

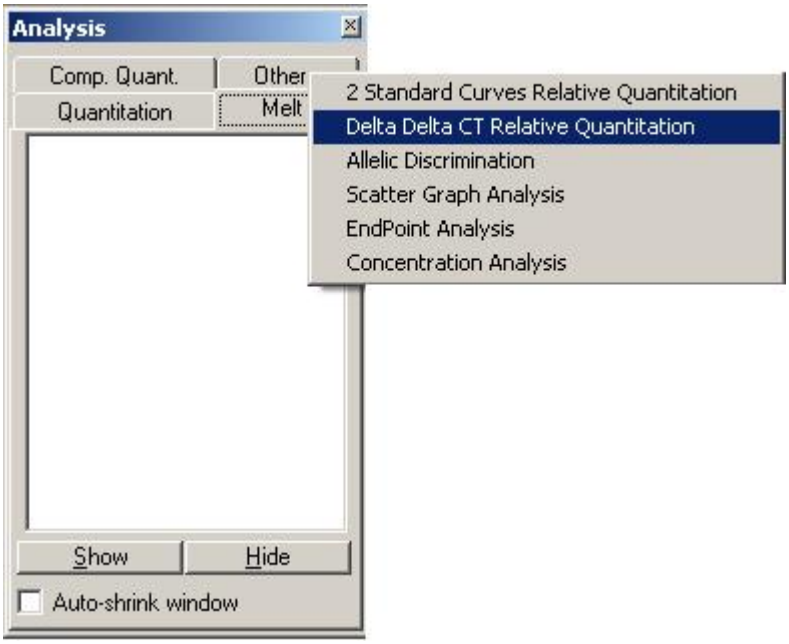
This method does not require the running of standard curves in each run. Each sample is first normalised for the amount of template added by comparison relative to the housekeeper gene (endogenous control). These normalised values are further normalised relative to a calibrator treatment. The calibrator is normally the wild-type, untreated control or time zero samples, for example.

It is essential however that the amplification efficiencies of the Gene of Interest and the Normaliser Gene are identical and that this is validated in accordance with the guidelines of this paper.

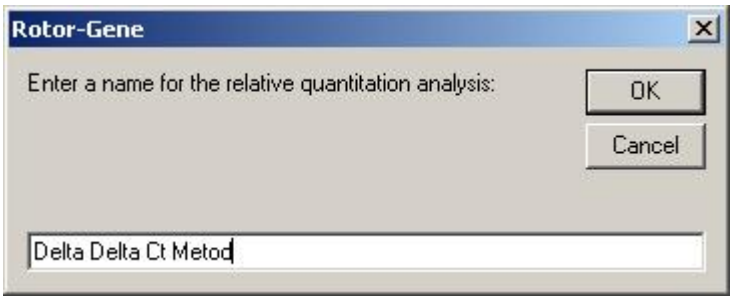
It is essential that the samples names are defined correctly in the Sample Editor, with the same samples labelled identically in each composite absolute quantitation analysis.

1. Analyse the data using Quantitation Analysis. Running a standard curve is not required by this analysis technique, as only the C_T values are used.

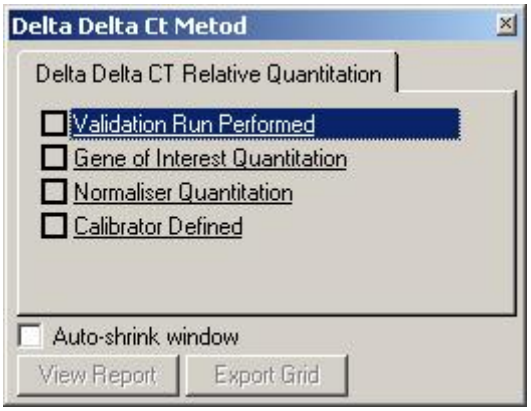
2. From the Analysis window **Other** tab select Delta Delta C_T Relative Quantitation. Select **New Analysis**.

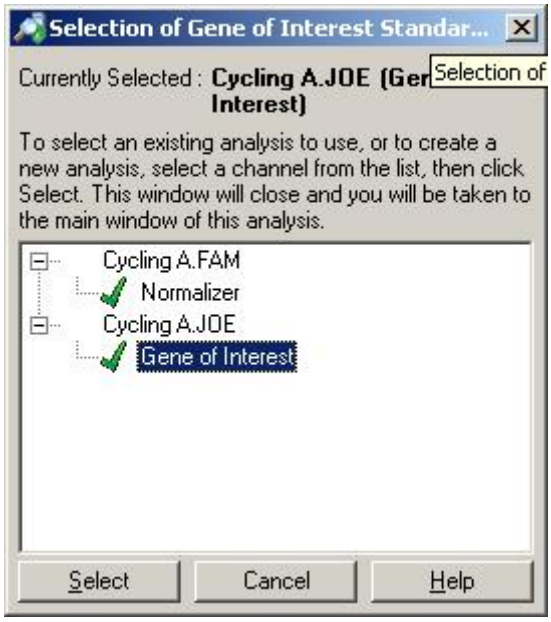


3. Enter a name for the analysis.

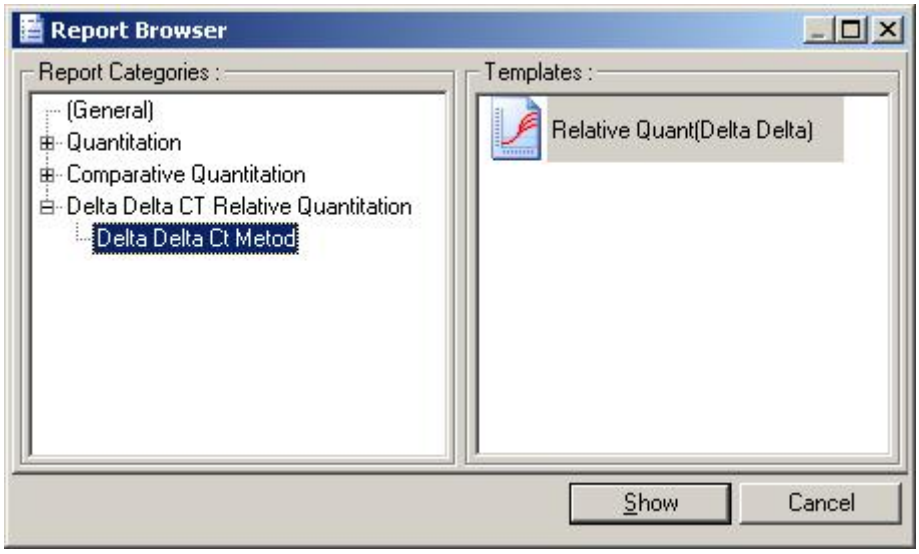


4. **Validation Run Performed** must be ticked to proceed with the analysis. Define the pages where the Gene of Interest and Normaliser Gene have been analysed. See the above paper for more information on the steps required for validation.

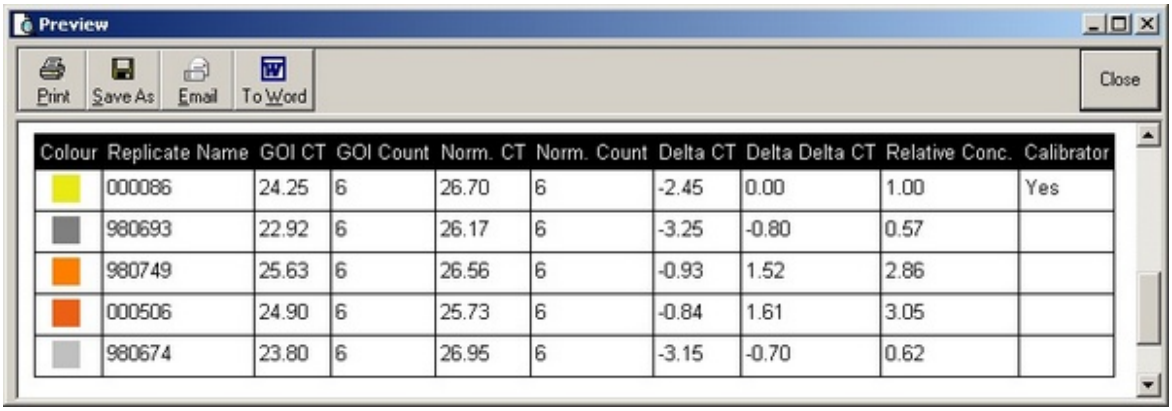




5. Click the **View Report** button to display the Report Browser. Select the analysis with the correct name from the Report Analysis. Click the **Show** button to display the Relative Quantitation report. The Export Grid option exports the results to an excel spreadsheet. If a calibrator is selected then the results are relative to the calibrator sample, which has a value of 1.



Example results are shown below. The CTs for the Gene of Interest analysis (GOI C_T), the Normaliser Gene (Norm. C_T), the Delta C_T, Delta Delta C_T and Relative Concentration (Relative Conc.) are displayed. The expression is relative to the Calibrator sample which is assigned a relative expression of 1.



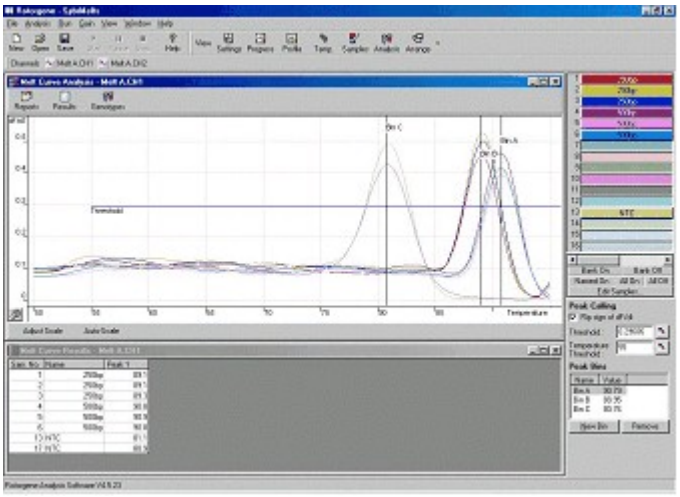
Colour	Replicate Name	GOI CT	GOI Count	Norm. CT	Norm. Count	Delta CT	Delta Delta CT	Relative Conc.	Calibrator
	000086	24.25	6	26.70	6	-2.45	0.00	1.00	Yes
	980693	22.92	6	26.17	6	-3.25	-0.80	0.57	
	980749	25.63	6	26.56	6	-0.93	1.52	2.86	
	000506	24.90	6	25.73	6	-0.84	1.61	3.05	
	980674	23.80	6	26.95	6	-3.15	-0.70	0.62	

6.6.5 Melt Curve Analysis

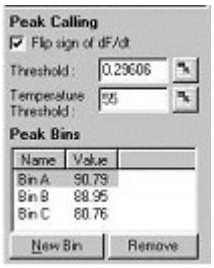
Melt Curve analysis analyses the derivative of the raw data, after smoothing. A common application of this analysis is genotyping or allelic discrimination. Peaks in the curve are grouped into bins, and all peaks below the threshold are discarded. One can then map bins to genotypes through the Genotypes command.

Typically after a cycling run has been finished a melt step can be added to visualize the dissociation kinetics of the amplified products. The sample temperature is increased at a linear rate and the fluorescence of each sample is recorded.

Below is a typical melt curve analysis shown for a SYBR Green I amplification.



Sidebar



Flip sign of dF/dT: Before defining peaks ensure the dF/dT sign is correct for the data set to give positive peaks.

Defining Peaks: In the Melt Curve Analysis, peaks can be defined and reported using different methods. One is to automatically call all the peaks for each sample. The other is to assign peaks to bins, which is useful for genotyping samples.

Any peak that is within a range of +/-2°C of the bin center will be assigned to the bin. If there are two peak bins close together then the peak will be assigned to the closest bin.

Bins are used to define the general area where you expect peaks to occur. The melt analysis software clusters peaks into bin groups, based upon actual peak values in the curve.

Note: The peak bins should not be visually positioned to estimate peak positions. Set the bins in the rough area of interest, then use the actual reported values in the results table for a more accurate result.

Peak Bins: To define a peak bin click on the **New Bin** button, then click and hold on the graph to define the center of the peak bin. To add another bin repeat the process or use the **Remove** button to delete peak bins.

Threshold Level: To set the threshold, click on the icon (grid with red arrow) then click and hold on the graph and drag a threshold line to the desired level.

Temperature Threshold: To set a temperature threshold, click on the icon (grid with red arrow) then click and hold on the graph and drag the threshold line to the right. This eliminates the threshold line for the lower temperatures.

Note: This is useful when there is noise on the signal at low temperatures.

Reports

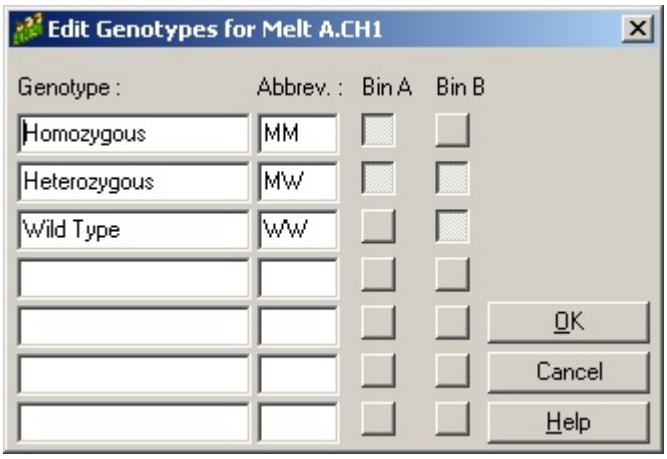
Opens the Melt Report selection window where you can choose a report to preview. You can generate a report based upon the currently selected channel, or you can generate a Multi-Channel genotyping report.

Results

Displays the result grid, showing peaks in samples.

Genotyping

Click on the genotyping toolbar and select the genotype definitions.



This screen lets you assign genotypes to the incidence of peaks in bins. The default genotype configuration is shown in the screenshot, with heterozygous samples having two peaks, homozygous samples a peak in the first bin and wild type samples a peak in the second bin. Next to the name of each genotype is a field for typing in an abbreviation. This is used when printing multi-channel genotyping reports so that all results from multiple channels can be read easily across the screen.

For multiplex analysis, genotypes must be set up in each channel. If, for example, a dual channel FRET analysis is run, where a wild type and heterozygous are expected in each channel, the setup procedure must be performed for each channel. The results will then be given in a multiplex report.

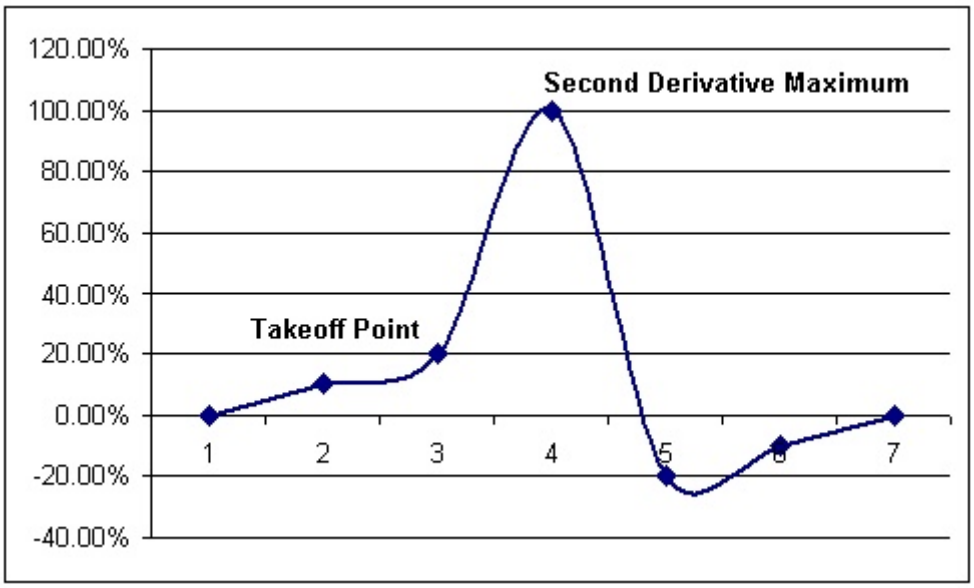
6.6.6 Comparative Quantitation

The feature "**Comparative Quantitation**" is used to compare the relative expressions of samples to a control sample in a run when a standard curve is not available. Users testing results from Microarray analysis frequently use this feature.

To perform the analysis, go to **Analysis** and select **"Comp. quantitation"**. Double-click on the channel to analyse. Chose a control sample by using the pull down menu on the right-hand side of the screen below the sample toggler. The table below the graph will automatically calculate the results.

The first column of the table shows the names of the samples. The second column is called "Takeoff" and gives the Takeoff point of the samples. The second derivative of the amplification plot produces peaks corresponding to the maximum rate of fluorescence increase in the reaction. The Takeoff point is defined as the cycle at which the second derivative is at 20% of the maximum level, and indicates the end of the noise and the transition into the exponential phase.

This graph shows a second derivative of a quantitation reaction, showing the relative positions of the second derivative peak and the Takeoff point:



The third column gives the efficiency of the particular sample. A 100% efficient reaction would result in an amplification value of 2 for every sample, which means that a doubling of an amplicon takes place in every cycle. In terms of the raw data, the signal should increase by a doubling amount in the exponential phase. So, if the signal was 50 at cycle 12, then went to 51 at cycle 13, it should go to 53 fluorescence units at cycle 14. All of the amplification values for each sample are averaged to produce the amplification value that is shown on the right-hand side of the screen. The more variation there is between the estimated amplification values of each sample, then the larger the confidence interval will be (indicated by the value after the ± sign). The confidence interval, for large N, gives a 68.3% probability that the true amplification of the samples lies within this range (1 standard deviation). By doubling the ± interval, one achieves a 95.4% confidence interval for large N.

Calibrator Replicate

As in the Comparative C_T Method (Livak 1991), the C_T of a calibrator sample is needed to measure relative to. In the version 6 software, this replicate is labelled the Control Replicate. Note that if multiple sample positions have been given the same name, the average of the Takeoff points of these samples will be used, allowing the operator to analyse using replicates of the calibrator. Ensure that you label replicates with identical names to use this feature correctly.

The average amplification is needed to calculate how much more or less a sample is expressed. If for example the amplification value was lower, a certain absolute copy number of an amplicon is obtained later than if the amplification value was higher. The last column finally gives the comparative quantitation value. Based on the Takeoff point and the reaction efficiency it calculates the relative concentration of each sample compared to the calibrator sample that was

chosen by the user. The number given is expressed in scientific notation.

Note: The value displayed to the right of the +- represents the standard deviation of the Average Amplification, after removal of outlier amplification values. If this value is large, then there may be a large error in the overall calculated concentration values.

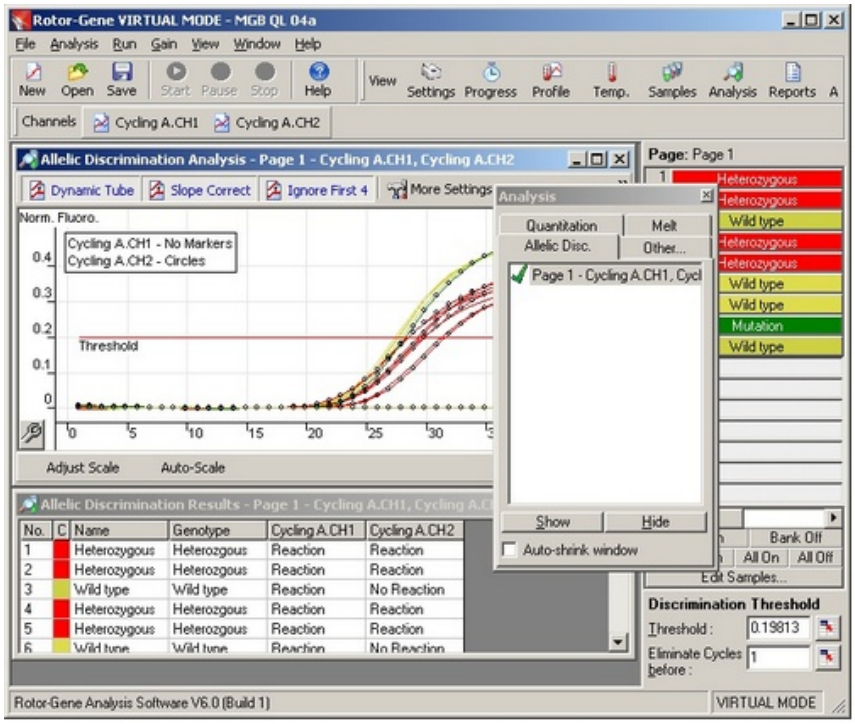
Steps taken to calculate relative concentrations:
1. The Takeoff points of each sample are calculated by looking at the second derivative peaks.
2. The average increase in raw data 4 points following the Takeoff is calculated and becomes the sample's amplification.
3. Outlier amplifications are removed to account for noise in background fluorescence.
4. The non-outlier amplifications are averaged to become a run "Average Amplification".
5. The average TakeOff point is calculated for all samples in the Calibrator Replicate.
6. The relative concentration for a sample is calculated as Amplification ^ (CalibratorTakeOff - ThisSampleTakeOff).
7. The result is displayed in scientific notation.

6.6.7 Allelic Discrimination

Allelic Discrimination uses real-time kinetic data from 2 or more channels to genotype samples.

For Allelic Discrimination, it is not sufficient to double-click on the channel you would like to analyze, as this analysis is performed using multiple channels simultaneously. To perform this analysis, either hold down SHIFT and click to highlight each channel you wish to analyze, or drag your mouse over these channels. Once the desired channels have been highlighted, click **Show**. The list will update to show all the channels on one line, with a tick next to them. This indicates that they are all being used in one analysis. You can "break apart" these channels by right clicking on the analysis and selecting **Remove Analysis...** You will then be able to include those channels in another Allelic Discrimination analysis. Please note that a channel can only be used once in each type of analysis.

Allelic Discrimination Analysis allows you to perform genotyping using dual labeled probes. The genotype of samples is displayed in the result window as follows:



Reports

Opens the Allelic Discrimination Report for preview.

Results

Displays the genotyping results spreadsheet. This spreadsheet is opened when the analysis is first displayed by default.

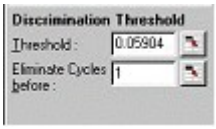
Normalisation Options

A variety of options are available to optimise the way in which the amplifcation plots are normalised:

- Dynamic Tube (Dynamic Tube Normalization)
- Slope Correct (Noise Slope Correction)
- Ignore First x (First x Cycles Noise Correction)

These terms are explained in the chapter Quantitation.

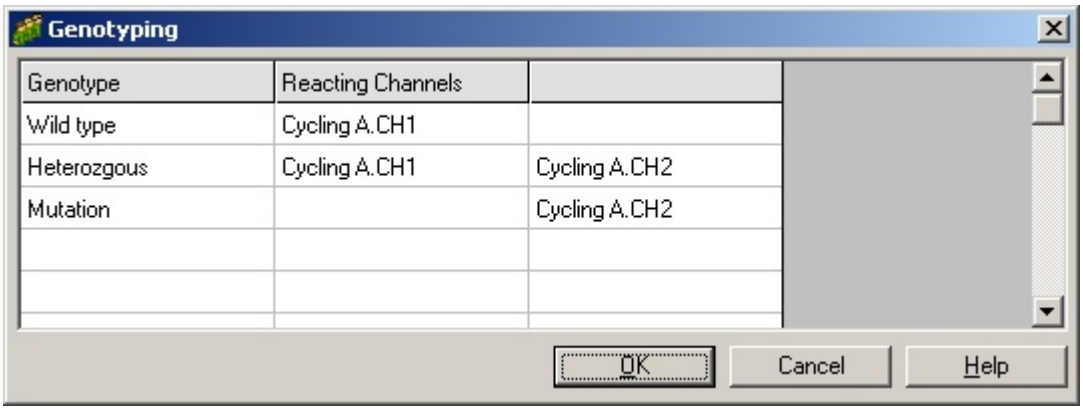
Discrimination Threshold



Discrimination Threshold: Enter values in these text boxes to position the discrimination threshold. All curves surpassing this line will be considered as having amplified for the purposes of genotyping. Click on the button to the right of each text box then drag the threshold on the graph to set these values visually.

Genotypes

Genotypes: Opens the genotype window to define which genotype is detected in which channel.

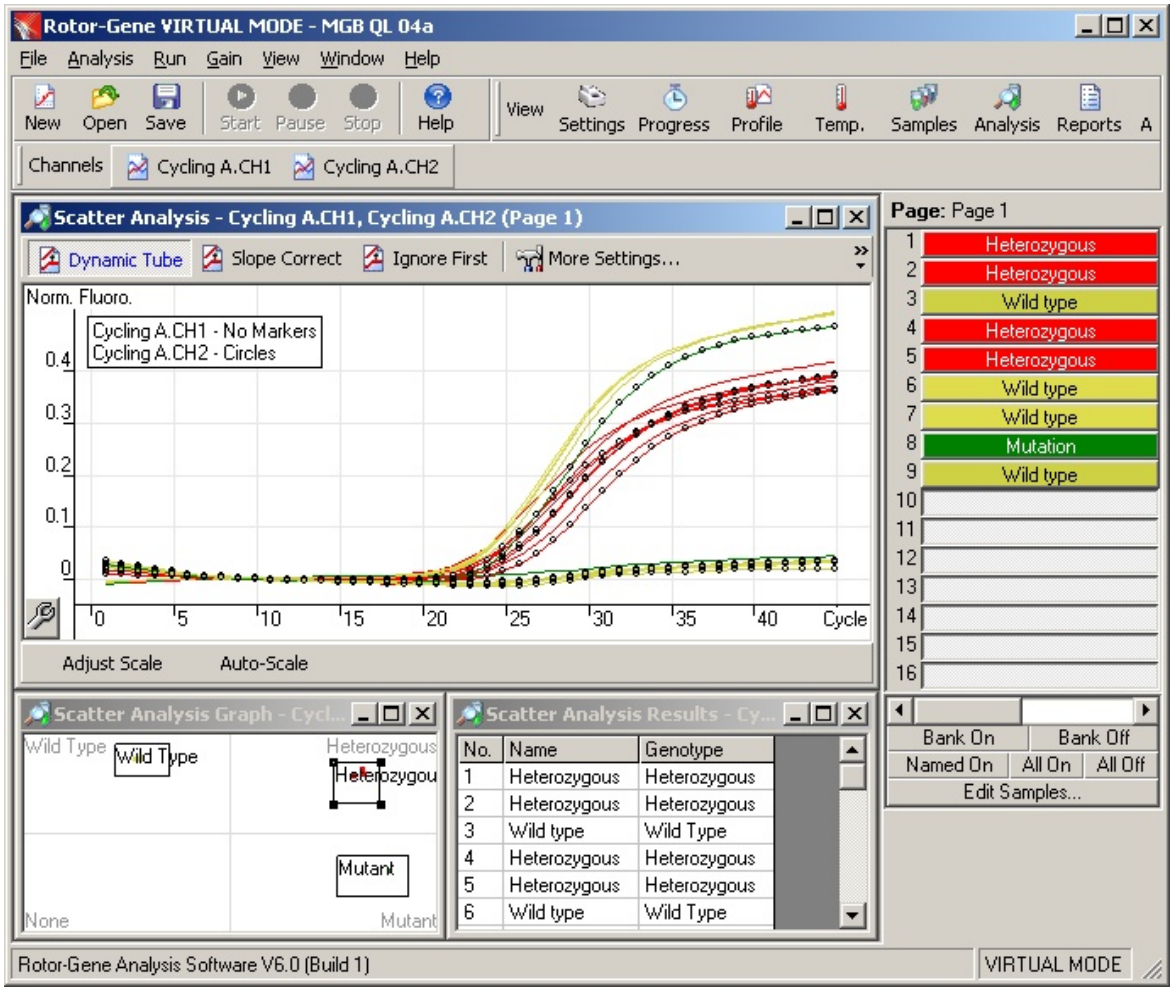


This screen lets you assign genotypes to reacting channels for an Allelic Discrimination analysis. In the above example, a sample is heterozygous if readings in channels Cycling A.CH1 and Cycling A.CH2 cross the threshold.

6.6.8 Scatterplot Analysis

For Scatterplot Analysis, it is not sufficient to double-click on the channel you would like to analyze, as this analysis is performed using multiple channels simultaneously. To perform this analysis, either hold down SHIFT and click to highlight each channel you wish to analyze, or drag your mouse over these channels. Once the desired channels have been highlighted, click **Show**. The list will update to show all the channels on one line, with a tick next to them. This indicates that they are all being used in one analysis. You can "break apart" these channels by right clicking on the analysis and selecting **Remove Analysis...** You will then be able to include those channels in another Scatter Analysis analysis. Please note that a channel can only be used once in each type of analysis.

Scatterplot Analysis allows for genotyping on the basis of the relative expression of amplification plots across 2 channels. Unlike Allelic Discrimination, the determination of genotype is made on the basis of regions defined on the scatterplot rather than a single threshold.



Reports

Opens the Scatter Analysis Report for preview.

Results

Displays the genotyping results spreadsheet. The genotype for each sample is determined by the regions defined by the user on the scatterplot.

Normalisation Options

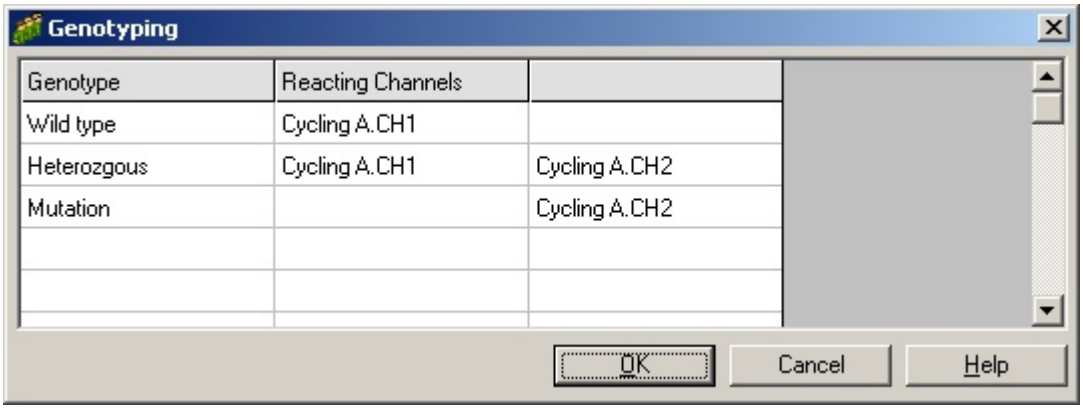
A variety of options are available to optimise the way in which the amplification plots are normalised:

- Dynamic Tube (Dynamic Tube Normalization)
- Slope Correct (Noise Slope Correction)
- Ignore First x (First x Cycles Noise Correction)

These terms are explained in the chapter Quantitation.

Genotypes

Genotypes: Opens the genotype window to define which genotype is detected in which channel.



On this screen, the user may assign genotypes on the basis of the channels on which a sample reacts. The channels selected will be used to label the corners of the scatterplot, and provide a guide to the user to the general area of the scatterplot in which regions should be defined.

Scatterplot

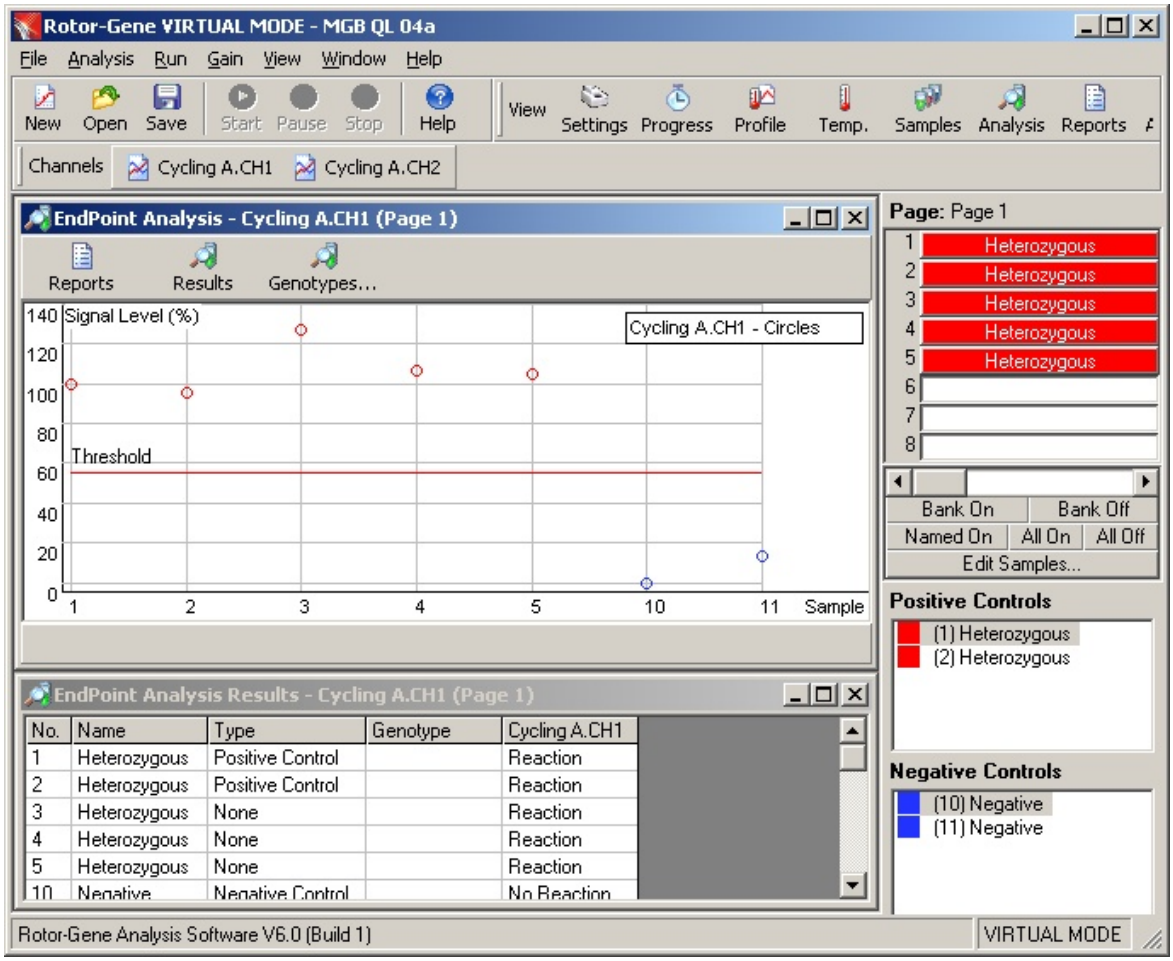
The scatterplot displays the relative expression of the two selected channels. The display is normalised to account for different fold increases in each channel, and log transformed to accentuate the differences in expression between samples.

To perform genotyping, the user defines regions by clicking and dragging a selection on the graph. The selection can then be labelled based on the genotypes configured in the Genotype window.

6.6.9 EndPoint Analysis

EndPoint Analysis is a technique which allows samples that amplify to be discriminated from non-amplifying samples at the end of a run. Results are qualitative (positive/negative), not qualitative. This technique is only suitable for endpoint data, and cannot be reliably performed using real-time amplification plots. For real-time determination of reactions, use the Allelic Discrimination or Scatterplot Analysis

Below is a screenshot of the EndPoint Analysis software module:



EndPoint Analysis is similar to Allelic Discrimination, in that the results are qualitative, and that names can be assigned to certain permutations of reactions over different channels. Where EndPoint Analysis is different is that only a single reading is available instead of a cycle-by-cycle reading for each sample. This means that the user must supply additional information to help facilitate the analysis, namely, the identification of **positive** and **negative controls**.

To facilitate the presentation of the amplification data, the signal levels are normalised relative to the known positives and negatives for each channel. The user then selects a percentage **signal level** as the reaction threshold.

Note: Endpoint analysis does not take advantage of the full amplification plot acquired through real-time thermal cycling, and is designed for post-run analysis of non real-time amplification. When possible, real-time qualitiative techniques such as Allelic Discrimination should be used when performing the cycling on a Rotor-Gene.

Terms Used In EndPoint Analysis

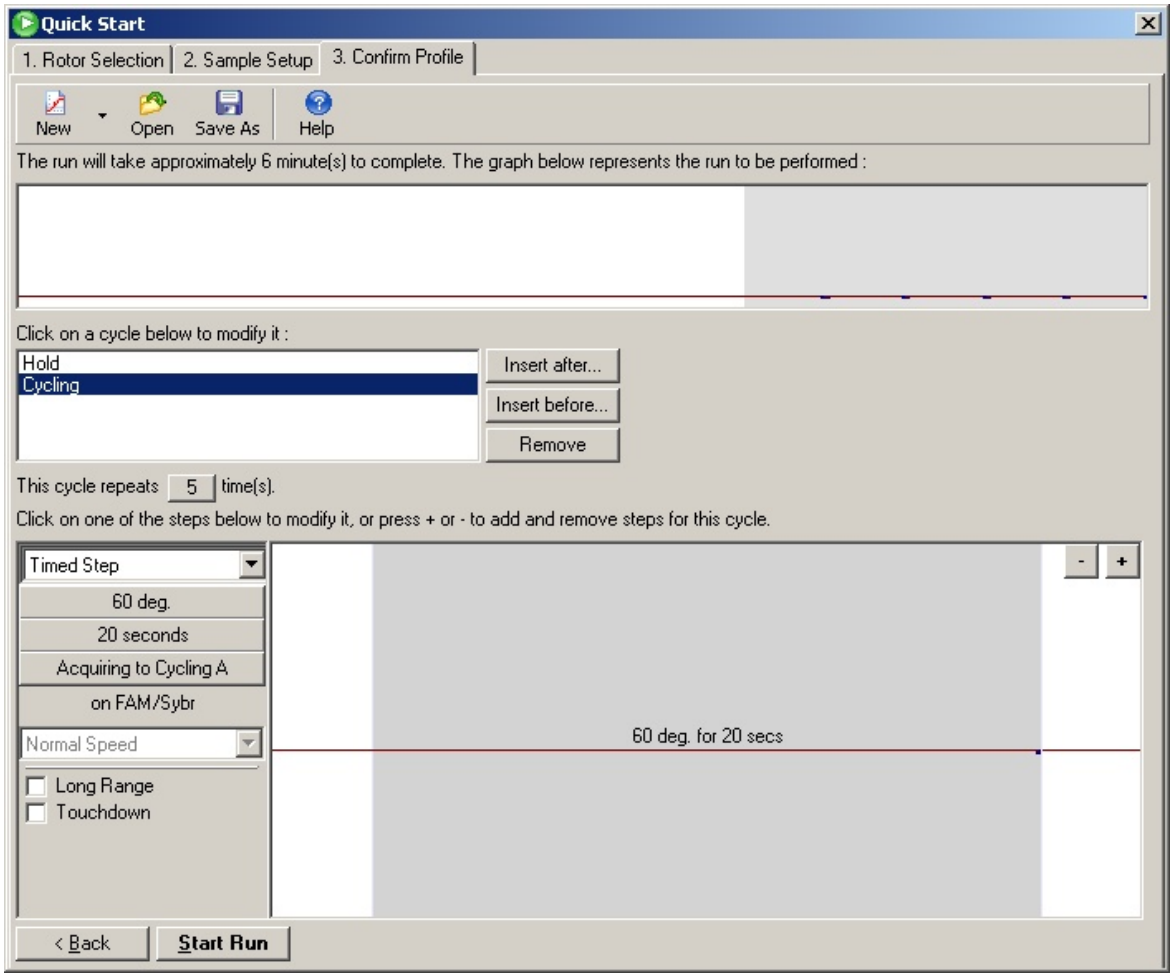
Below are explanations of the terms and concepts used in EndPoint Analysis:

Term	Explanation
Positive Control	A sample which is known to amplify and produce a positive result.
Negative Control	A sample which is known not to amplify. This represents the typical background signal.
Threshold	A signal level above which a sample is said to be positive (amplified). This setting must be adjusted by the user for each run.
Signal Level	A percentage of fluorescent signal, normalised so that the signal of the highest positive control is 100% and the lowest signal of the negative controls is 0%.
Genotype	An interpretation of different permutations of reactions on different channels. For example, one could assign a genotype of "Heterozygous" to samples which reacted in both channels "FAM/SYBR" and "JOE". The genotype can also be used for reporting results of reactions with internal controls. For example, you may wish to report results such as "Inhibited", "Positive", "Negative" on the basis of whether a reaction was seen in certain channels.

Profile Configuration

To perform an EndPoint analysis, you should first amplify the samples in a thermal cycler.

Once this has finished, transfer the tubes to the Rotor-Gene, and perform a profile with a hold at 60 degrees for several minutes, then a cycling step with 1 step, on 60 degrees for 20 seconds, acquiring on the required channel. Set the number of repeats to 5, as shown below:



Please note that these times are a guide only, and may vary for your particular application.

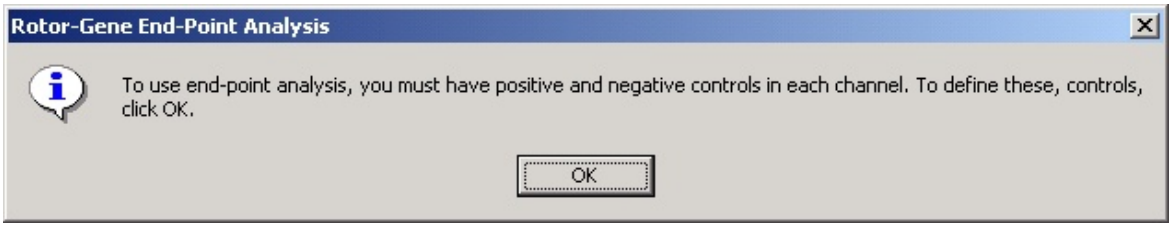
The more repeats in the profile, the more information will be available to perform the analysis. The analysis module will automatically average all the readings taken into a single value for each sample. There is no set number of repeats required, and unless you are performing a run where a very fine level of accuracy is required, 5 repeats should be largely sufficient.

Analysis

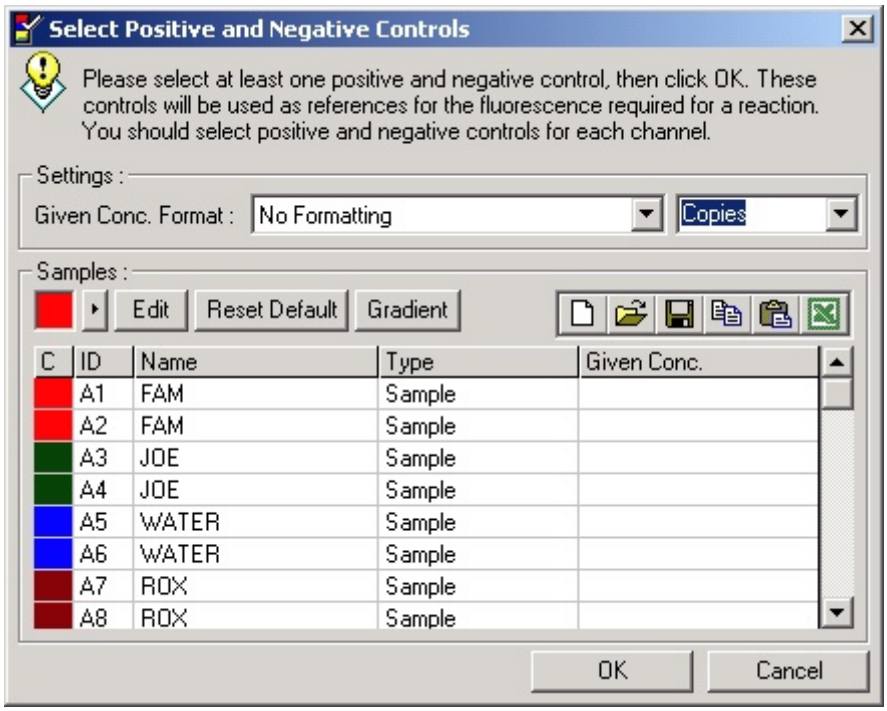
You can perform EndPoint analysis on a number of channels simultaneously. To create a new analysis, click on the EndPoint tab, select the channels by dragging over them with your mouse, and then click **Show**. The EndPoint analysis window will be displayed.

Define Controls

When you open an EndPoint analysis for the first time, you will likely not have set up your Positive and Negative controls. The following message will be displayed :



After clicking OK, the sample editor appears, allowing you to define Positive and Negative controls. To set a sample to be a Positive or Negative control, click on the cell containing the sample's type, then select the relevant control type from the drop-down box which appears next.



This screen functions in an identical manner to the **Sample Editor**.

Normalisation

Normalisation of the EndPoint data scales all signal levels to be within the range of 0-100%. You must have selected at least one **Positive Control** and one **Negative Control**, however, you will need more than this if you are analysing multiple channels and your standards are not multiplexed. You should also use more than one control of each type if there is the risk that your positive control may not amplify.

1. For each channel, all the Positive Controls are sampled and one with the highest fluorescence is set to be 100%. This allows for a Positive Control to fail without affecting your run, if you are running duplicate controls.
2. All the Negative Controls are then sampled, and the one with the lowest fluorescence level is set to 0%.
3. The raw fluorescence values of the other samples are then scaled relative to the highest Positive Control and the lowest Negative Control.

Here is an example:

Sample	Type	Fluorescence
1	Positive Control	56.3
2	Positive Control	53.0
3	Negative Control	4.5
4	Negative Control	4.3
5	Sample	48.1
6	Sample	6.4

This run was a success, as the two positive and negative controls are both close together, and are outside the fluorescence values of the samples.

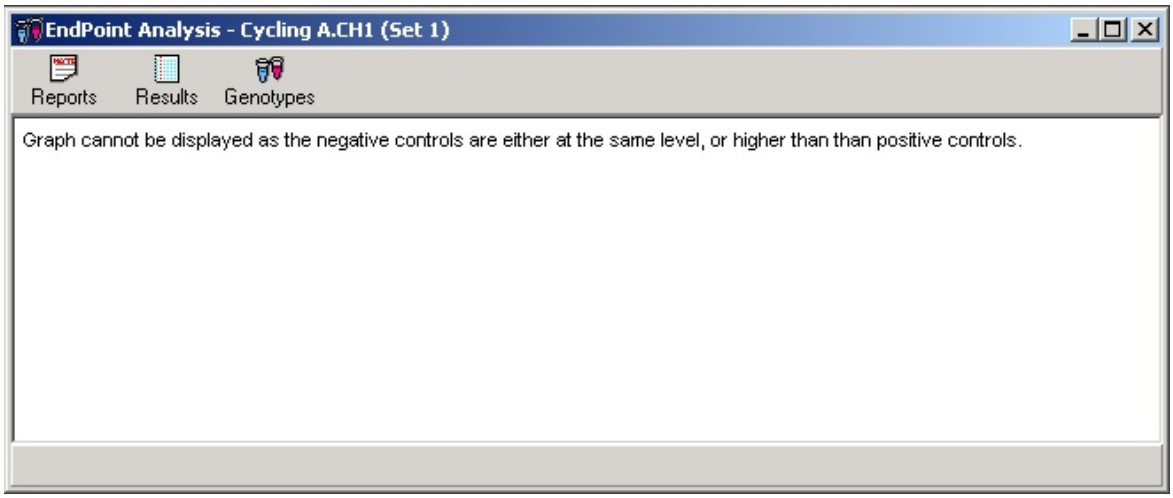
Here are the normalised values:

Sample	Type	Expression(%)
1	Positive Control	100.0
2	Positive Control	93.7
3	Negative Control	0.4
4	Negative Control	0.0
5	Sample	84.2
6	Sample	4.0

As Sample 1 was the Positive Control with the highest fluorescence, it was set to 100% expression, with the other positive control slightly lower. Sample 4, the lowest Negative Control was set to 0% expression. It is now obvious that Sample 5 is more likely to have amplified, whereas Sample 6 is likely not to have amplified.

NOTE: If Positive and Negative Controls are not selected carefully, it is possible to achieve expression levels of greater than 100%, and lower than 0%. This can be interpreted as "the sample expressed itself more than the positive controls", or "it is less likely that the sample reacted than that the negative controls reacted". Such a result is not of concern as the purpose of the analysis is obtain a qualitative answer.

If you receive a message stating that the Negative Controls are higher than the Positive Controls, then you have incorrectly set up your samples. For example, your Negative Controls had a level of 10Fl, but the Positive Controls had a level of 5Fl. Since this is an illogical result, you will receive the following warning:



Normalisation on Multiple Channels

It is possible to analyse signal data over multiple channels, however, the sample setup is a little more complex. EndPoint analysis assumes multiplexing and so each tube can only have a single tube position. This can cause difficulties when a sample position is a positive control for one channel, and a negative control for the other. It is not possible to analyse such a setup in the software at this stage.

Although the samples definitions operate from a single sample definition, the normalisation occurs independently for each channel.

If a tube position is a positive control for at least one channel, mark its type as Positive Control. Otherwise leave it as type Sample. The same applies for Negative Controls.

As an example, if a sample is a Positive Control on FAM/SYBR, but not on JOE, the sample should still be defined as a Positive Control. So long as there is least one Positive Control on JOE which will amplify, the definition of the sample as a control for FAM/SYBR will be ignored.

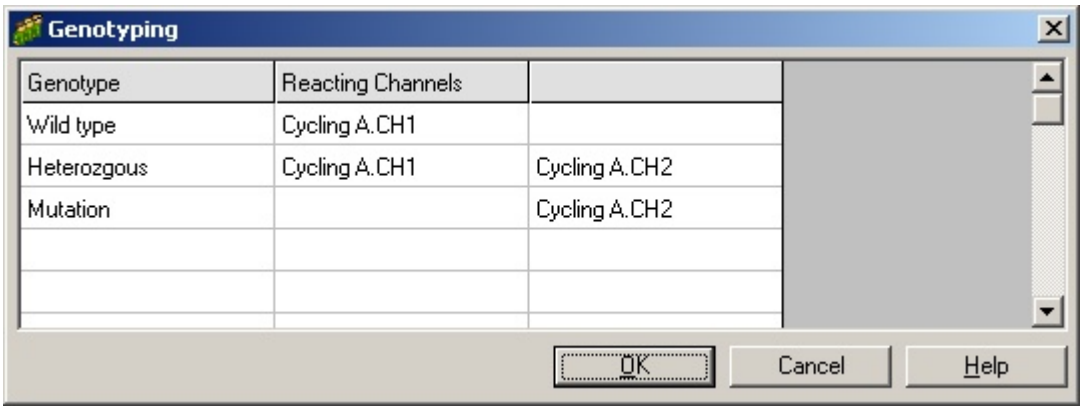
Threshold

The **Threshold** is used to determine the percentage level of expression required for a reaction on each channel. Once the **Positive** and **Negative Controls** have been defined, all channels will be normalised to the same scale, 0-100%. This is the reason why only one threshold is needed, even when analysing multiple channels.

Click and drag the threshold line to an area between 0-100%. Your threshold should not be too near samples on either side of the line as this indicates that the run was not conclusive. If the difference between a sample amplifying or not is a matter of a few percent, then the same reaction run again could appear on the other side of the threshold.

Genotypes

Genotypes: As in Allelic Discrimination, this option opens the genotype window to define which genotype is detected in which channel.



This screen lets you assign genotypes to reacting channels. In the above example, a sample is heterozygous if readings in channels Cycling A.CH1 and Cycling A.CH2 cross the threshold.

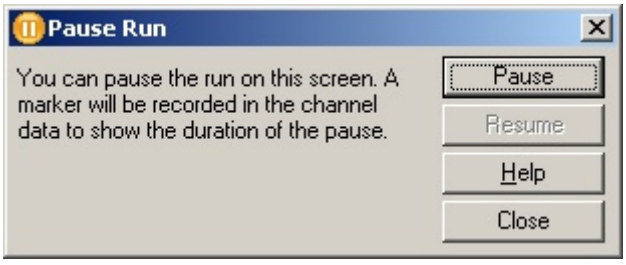
6.7 Run Menu

6.7.1 Start Run

Starts the defined Temperature Profile with the current Gain settings. Before the Rotor-Gene starts the Profile Run Confirmation screen is shown. A graphical representation of the temperature profile to be run is displayed along with the Gain settings for each channel.

6.7.2 Pause Run

You can use this screen to pause and resume a run. As performing this operation can seriously affect the results of a run, a marker is drawn across all channels acquired to by the currently running cycle. A message is also placed in the message screen.



Caution: When pausing the machine, it will not be cooled completely to room temperature. You should exercise caution before handling the rotor or any tubes in the machine.

6.7.3 Stop Run

When stopping a run you will be prompted to confirm your intention.

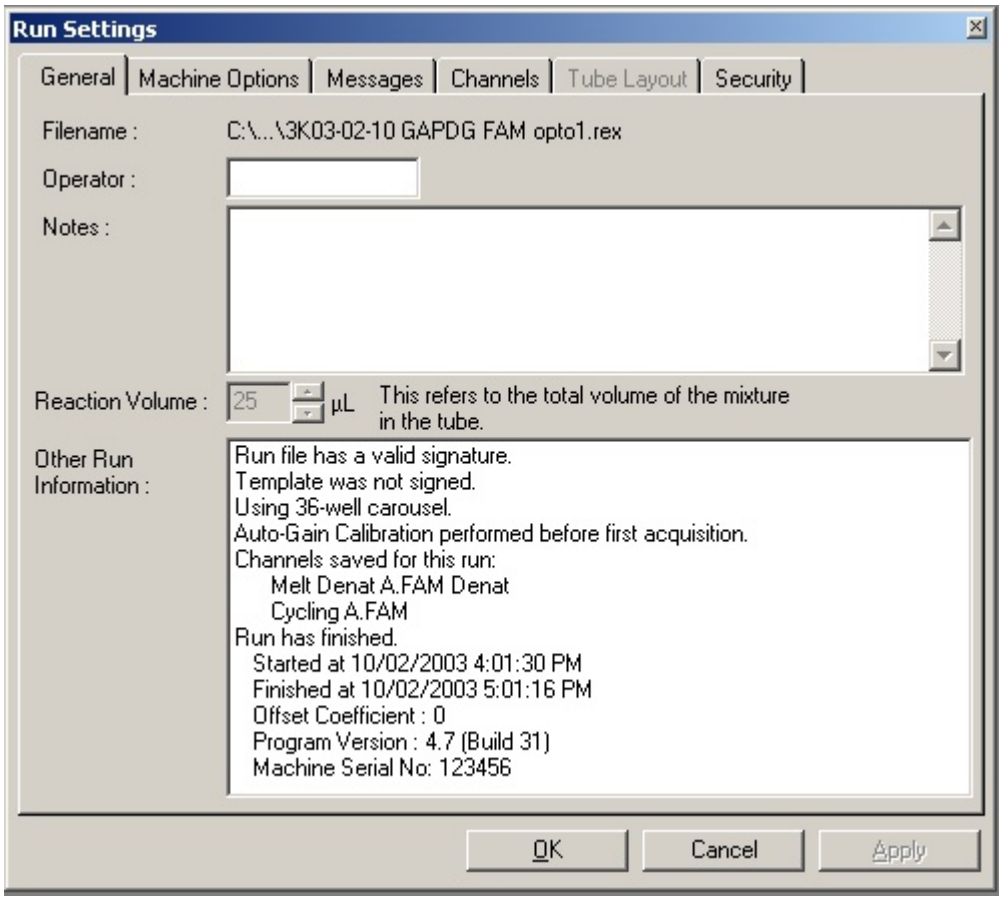
6.8 View Menu

6.8.1 Run Settings

General

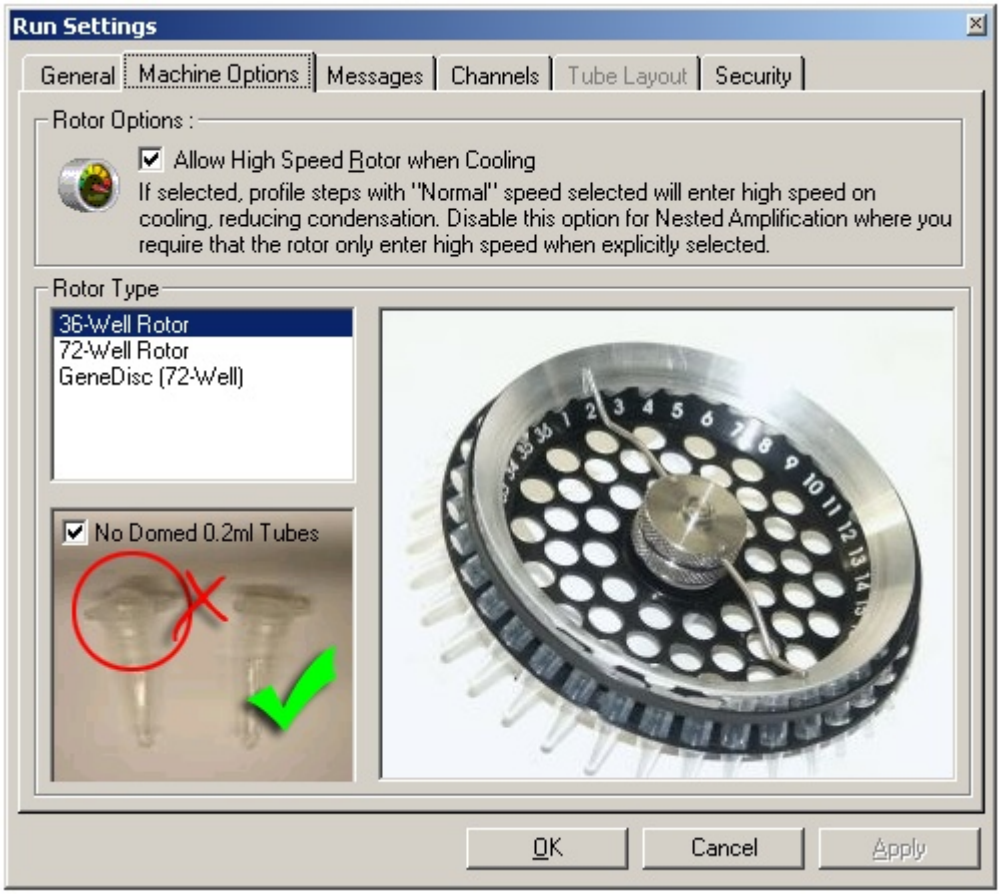
This window allows the setup of run information, run filename, analysis date, operator and any associated notes.

The run information window contains all information except for the profile required to configure a run. The following information is displayed after a run has finished: type of machine, Gain settings, number of channels and time of start/finish.



Machine Options

This tab contains settings for the configuration of the Rotor-Gene instrument.



Allow High Speed Rotor when Cooling: When enabled, the rotor will spin more quickly when the machine is cooling. This option is useful as the high speed firms the tubes in their places and prevents them from moving around. This option should generally be left on. You may need to disable this if you are using an advanced technique where you require direct control over the rotor speed.

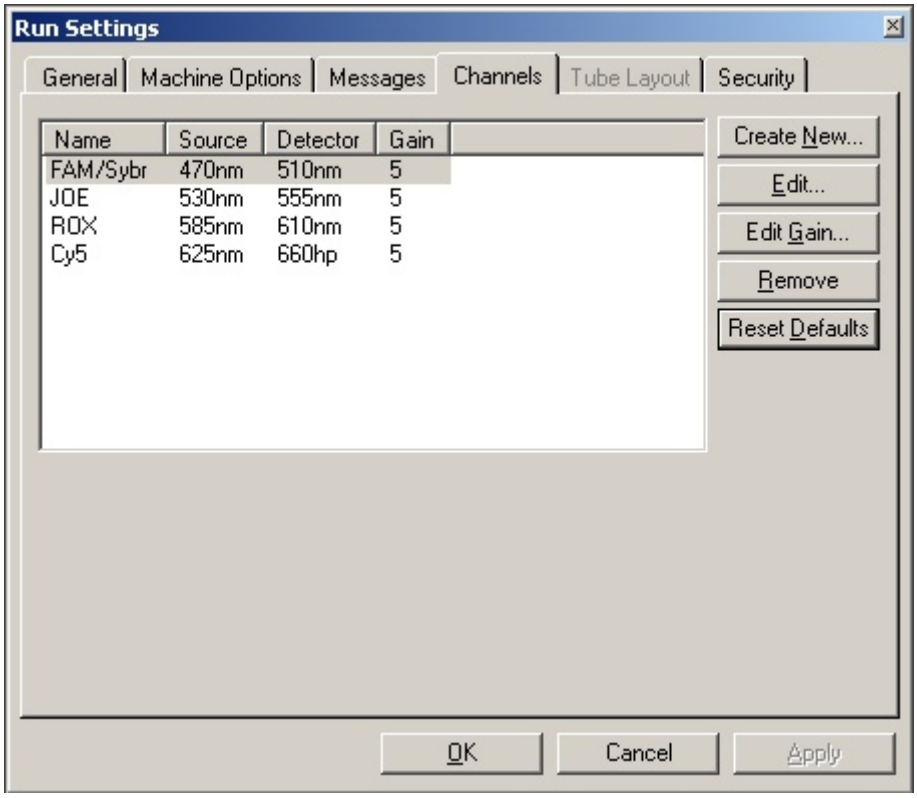
Rotor Type: The rotor should be set to that currently installed in your Rotor-Gene. If opening an existing run, this setting will reflect the rotor that was installed in the machine at that time. The options available depend on your machine and the rotors it supports.

Messages

This tab displays messages where the operator has changed functions such as pausing the machine or skipping cycles during a run. It also displays warnings received during the run. You should check this tab if you receive unusual results.

Channels

If configuring a new run, the channels tab displays the current configuration of the channels available in the machine. If you are viewing an existing run, then the information displayed represents the configuration of the channels when the run was performed. You can modify the Gain and create and modify channels if you are using a Multi-Channel system. To restore default channels if a run corrupts your machine's channel settings, click Reset Defaults.



Name: Describes the name of the channels.

Source: Specifies the excitation wavelength of the source LED.

Detector: Specifies the detection wavelength and filter type. (nm=band pass, hp=high pass)

Gain: Specifies the Gain for that particular channel.

Create new: This feature allows creating new channels. Pressing this button opens a window, which asks for a new name, source and detection filter. The filters can be chosen by using the pull down menu next to each window.

Some commonly used channel configurations are listed below:

Channel Name	Source/Detector	Example Fluorophores
FAM/SYBR	**470nm/510nm**	FAM, SYBR Green I, etc
JOE	**530nm/555nm**	JOE, VIC, TET, etc
ROX	**585nm/610nm**	ROX, Tamra, etc
Cy5	**625nm/665nm**	Cy5, LC 640, etc
User defined	**470nm/585hp**	SYBR Green I and others
User defined	**470nm/610hp**	FRET, Cy5, LC640 and others

Channels FAM/SYBR, JOE, ROX and Cy5 are standard configurations for 4 channel multiplex detection. Quencher molecules need to be used on the 3' end of the dual labeled probes so as not to occupy spectral bandwidth. The best combination is BHQ (black hole quenchers from Biosearch Technologies) FAM/BHQ1, JOE/BHQ1, ROX/BHQ2, Cy5/BHQ3.

Channel 470/585hp can be used for single channel SYBR Green I detection and gives more sensitivity than the FAM/SYBR channel, due to the use of a high pass filter.

Channel 470/610hp is used to detect eg. Cy5 or LC640 in a FRET reaction where an increase in energy is detected. This can be used for quantitation or mutation detection.

NOTE: For mutation detection using FRET probes, the information is contained in the differential of the melt curve and it makes no difference whether the increase in Cy5 (FRET) or the decrease in FAM (quenched FRET) is monitored. This is helpful when looking at multiplexing a mutation detection assay as a FAM/BHQ1 and JOE/BHQ1 probe sets (quenched FRET) can be used in conjunction with FAM/SYBR and JOE Channel configurations.

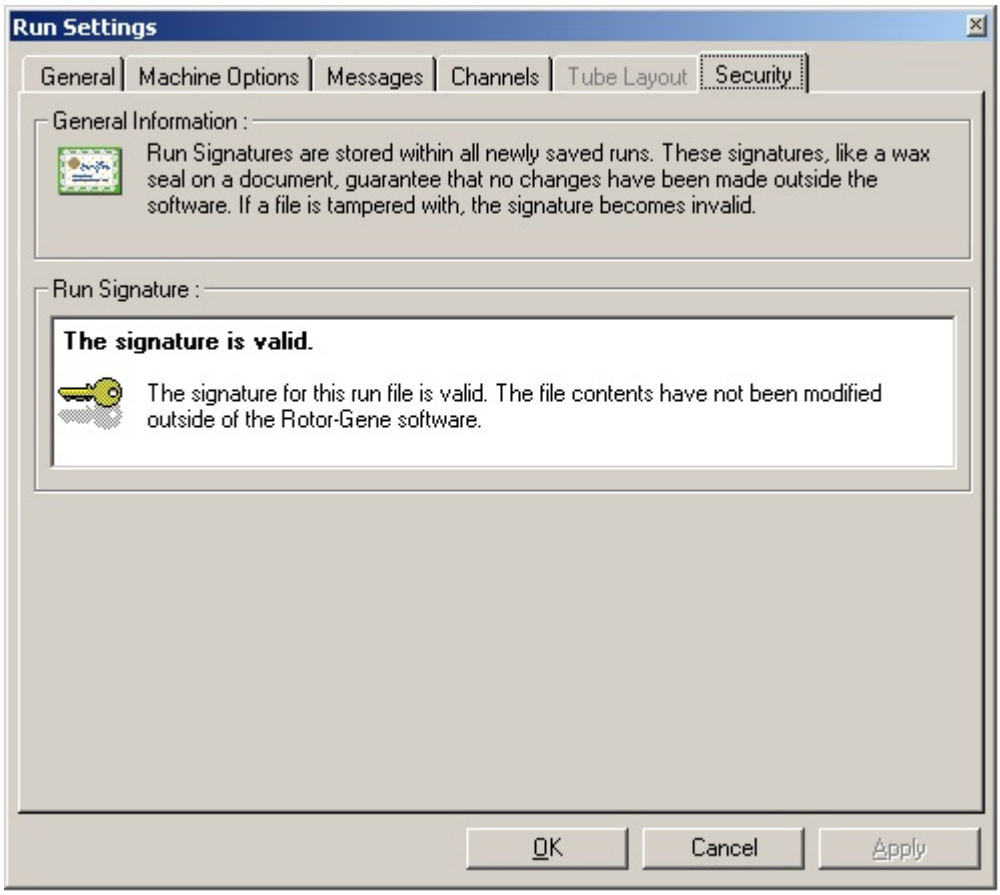
Tube Layout

Tube Layout: If you are using a 72-well system, you may wish to reorganize samples to more closely match the labeling that you are using on a 9x8 block. The tube layout tab allows you to label samples sequentially (the default), so that samples are listed in the order they are placed in the machine, or by every 9th sample (1A, 1B, 1C, etc). This is useful when loading samples with a multi-channel pipette.

If modifying this setting, you should perform a test run to confirm that the organization option you have selected works in the manner that you expect.

Security

The security tab displays information about the Run Signature. The Run Signature is a non-reversible key which is regenerated every time the file is changed. If any section of the REX file is modified outside of the software, the signature and the file will no longer match. Using the signature, you can ensure that the raw data is not modified outside the application, that the profile has not been tampered with and that the temperature graph is valid. The signature also protects against non-malicious corruption, such as file-system errors.

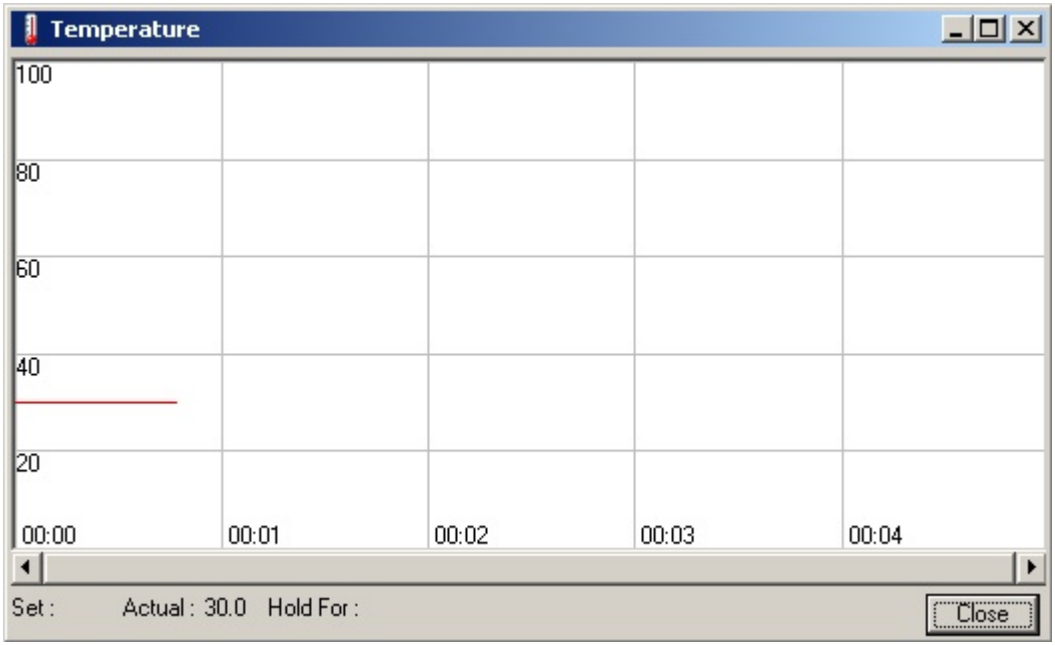


Important: The Run Signature only exists in recent versions of the software. The absence of a signature in old files does not mean that a file has been modified. However, any new runs you create should contain a signature and so can be validated. You should treat with suspicion any file where the signature is invalid.

You can ensure security for your files by making a laboratory policy that all run files must be run using 5.0.8 or later. Runs performed in this version will display a warning if they have not been signed, whereas older runs will not. However, all reports will include the version number of the software, so you can also treat as suspicious newly run files on old versions of the software.

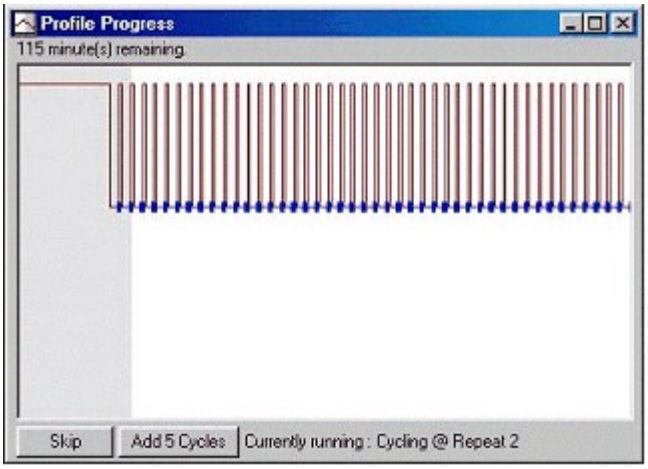
6.8.2 Temperature Graph

The temperature graph shows the temperature of the samples during a run. As the run proceeds the Set, Actual and Hold time is shown for each step of the program. When loading an existing run file, the temperature graph shows the temperature history during the run. The vertical scale is 20-100°C and the horizontal scale is shown in minutes. Use the scrollbar to scroll backwards and forwards throughout the temperature history.



6.8.3 Profile Progress

This screen shows a graphical representation of the thermal profile associated with the run. When performing a run, the shaded portion of the window indicates the number of cycles that have been completed. There is also an estimate of how many minutes the program will take to finish this run.



There are two further buttons on the window: **Skip** and **Add 5 Cycles**. While skip allows skipping any steps of the profile, the "Add 5 Cycles" button allows adding five repeats to the current step.

6.8.4 Edit Samples

This window has identical functionality to the sample edit grid in the New Run Wizards, except that the toolbar functions are also available in the File and Edit menus. This menu can also be accessed by right clicking over the sample list on the right side of the main screen.

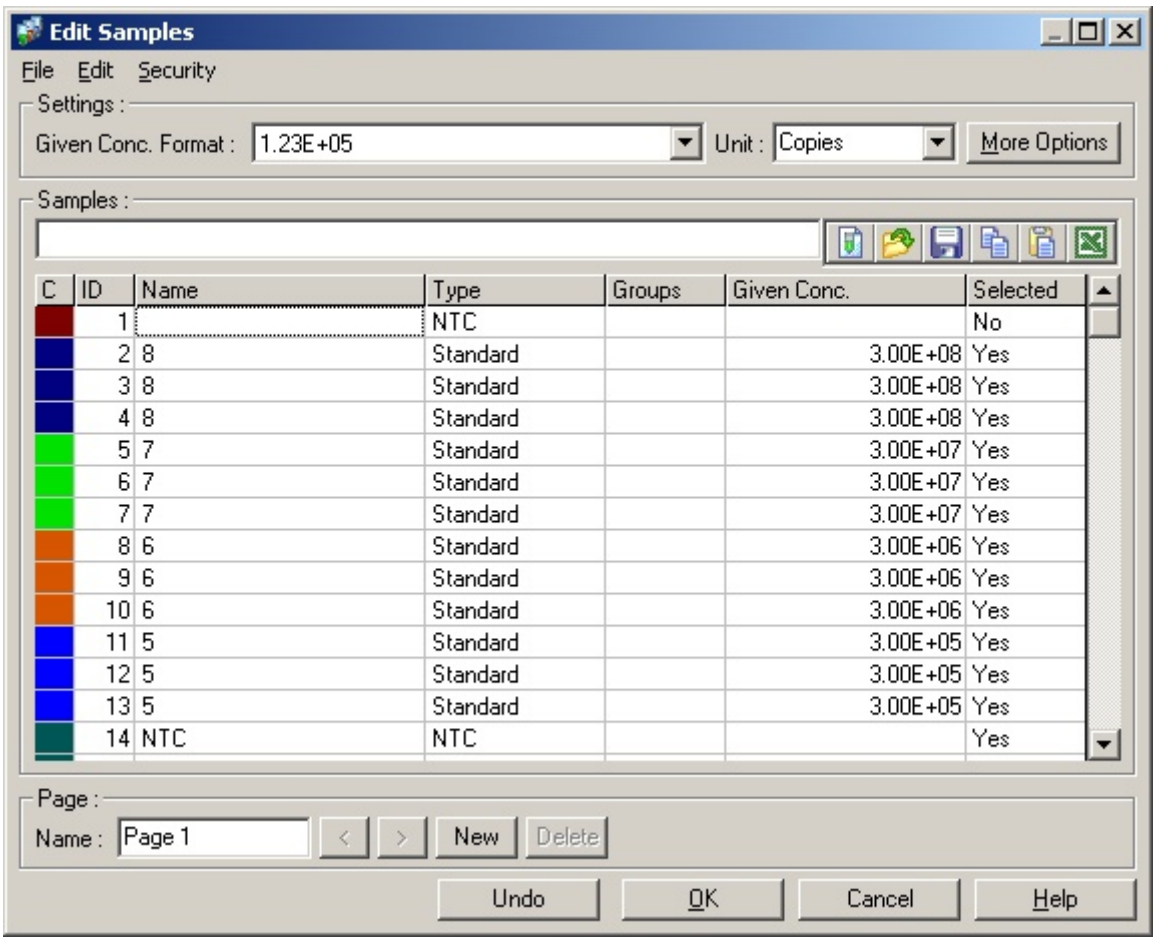
Three menus are given on the top of the screen, **File**, **Edit** and **Security**. The file menu is used to create a new (blank) sample sheet, open an existing sample template or save sample names as a template for future use. The extension of those files is *.smp. The edit menu allows you to copy and paste rows in the grid. The security menu allows you to lock the sample definitions.

Given concentration format: This drop down menu is used to choose a suitable format for the concentration display. Concentrations are automatically formatted in a manner appropriate with your currently selected locale.

Unit: This drop down menu sets the units of measurement for your assay.

Edit: Pressing this button opens the colour selector. It is possible to select multiple rows when assigning tubes a colour.

Reset Default: Click this to reset all selected color cells back to their default color values.



Gradient: The gradient function allows choosing a gradient from the first to the last selected color. Several gradients can be defined in a sample setup.

Tab Key: The Tab key can be used to navigate around the sample editor.

New Icon: Clears the sample grid in preparation for data entry.

Open Icon: Brings up a dialog box in which you can select a Rotor-Gene Sample file to import. Note: A 32-well sample setup cannot be imported into a 72-well setup and vice versa. The number of samples in the open sheet and the file being imported must match.

Save As Icon: Brings up a dialog box in which you can enter the name and folder in which to save a copy of the current sample definitions.

Copy Icon: Copies the selected cells.

Paste Icon: Pastes cells which had been selected with the copy command onto the currently selected position on the grid.

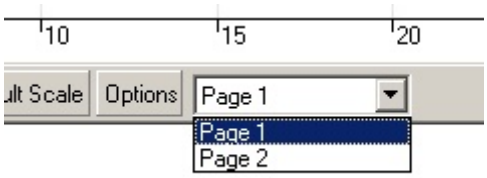
Excel Icon: Pressing the Excel icon prompts you for a file name. Excel is than opened automatically with the grid contents.

Sample Types: Samples can be defined as one of several types, listed below:

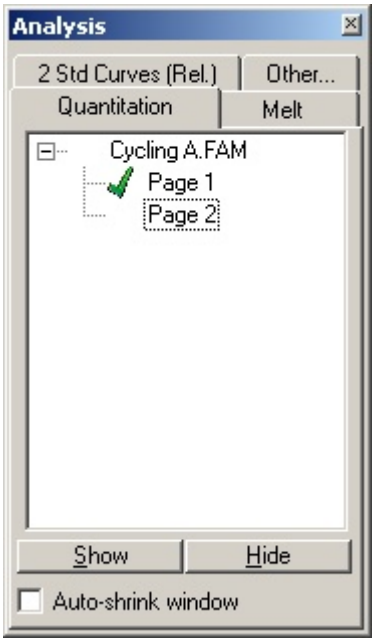
Sample Type	Meaning
None	No sample in that position.
NTC	No Template Control
-ve Control	Negative Control
+ve Control	Positive Control
Unknown	Unknown sample to be analysed.
Standard	Values are used to construct a standard curve to calculate unknown sample concentrations.

Sample Pages: This new function allows the user to have different sample definitions, and indeed, separate experiments in the same run. This is useful if you are analysing different products in different channels. Use the arrow buttons to move between the sample pages. Use **New** and **Delete** to create and delete pages. You can also have multiple sample definitions for the same channel, if you wish to run multiple standard curves without multiplexing. Simply define the samples of interest and their related standard curves on separate pages. You will be able to analyse the single channel twice with each set of definitions independently. Sample pages do not need to be labelled "Page 1" -- they can be given any name, such as "Housekeeper". This name will then appear in reports.

When viewing the raw amplification plots, you can select which sample definitions to use to display by the drop-down selector next to the Options button:



You can also select the page to use when performing an analysis:



Given Conc.: Shows the concentration for each of the standards defined. The units can be defined as a decimal or log number. If a dilution series has to be typed in it is only necessary to type the first two standards. By pressing ENTER, the program automatically adds the next logical dilution in the series, if there are further Standards defined below.

Line Style: You can modify the style of the line to improve readability of graphs on black and white printers. You can also add emphasis to certain lines by modifying their style. To access this feature, click on the right arrow button next to the Edit button:

The toolbar will then change to show the default style "Solid". You can change this to "Dashed", "Dotted" or a number of other possibilities. When you have finished, click the left arrow button to return to the Edit, Reset Default and Gradient view.

Productivity Tips:

Multiple-Row Entry: If you need to enter the same information for several rows at once, select all the rows, then begin to type. The information will be entered into each row. This works for selecting sample types, choosing colors or entering concentrations.

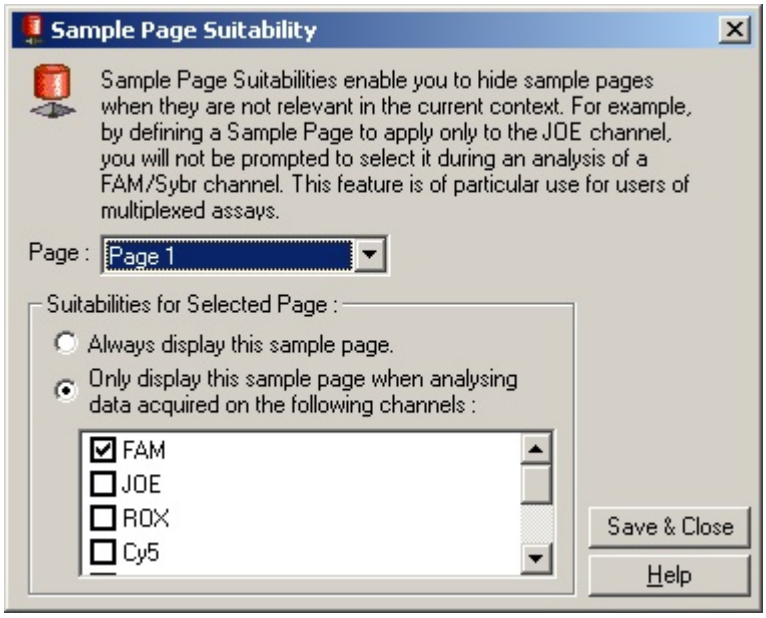
Sample Type Hotkey: To quickly select a sample type, just enter the first letter of its name. So, to set 5 samples to be Non Template Controls, select them in the sample type column, then press N for NTC. All samples will be converted to NTC's.

Note: A complete sample description can be saved as a sample file (*.smp) and loaded into future runs with the same sample configurationn.

Suitabilities

Sample page suitabilities allow users to match sample pages to channels. In a SYBR Green assay, for example, all page definitions are applicable to the FAM channel, since there is only a single setup. In a relative quantitation assay, however, the sample page for the Gene of Interest may apply to the FAM channel, and the Housekeeper to the JOE channel. In this instance, setting up Suitabilities reduces the number of analysis options available to those that make sense for the particular assay.

The sample suitability screen is shown below:

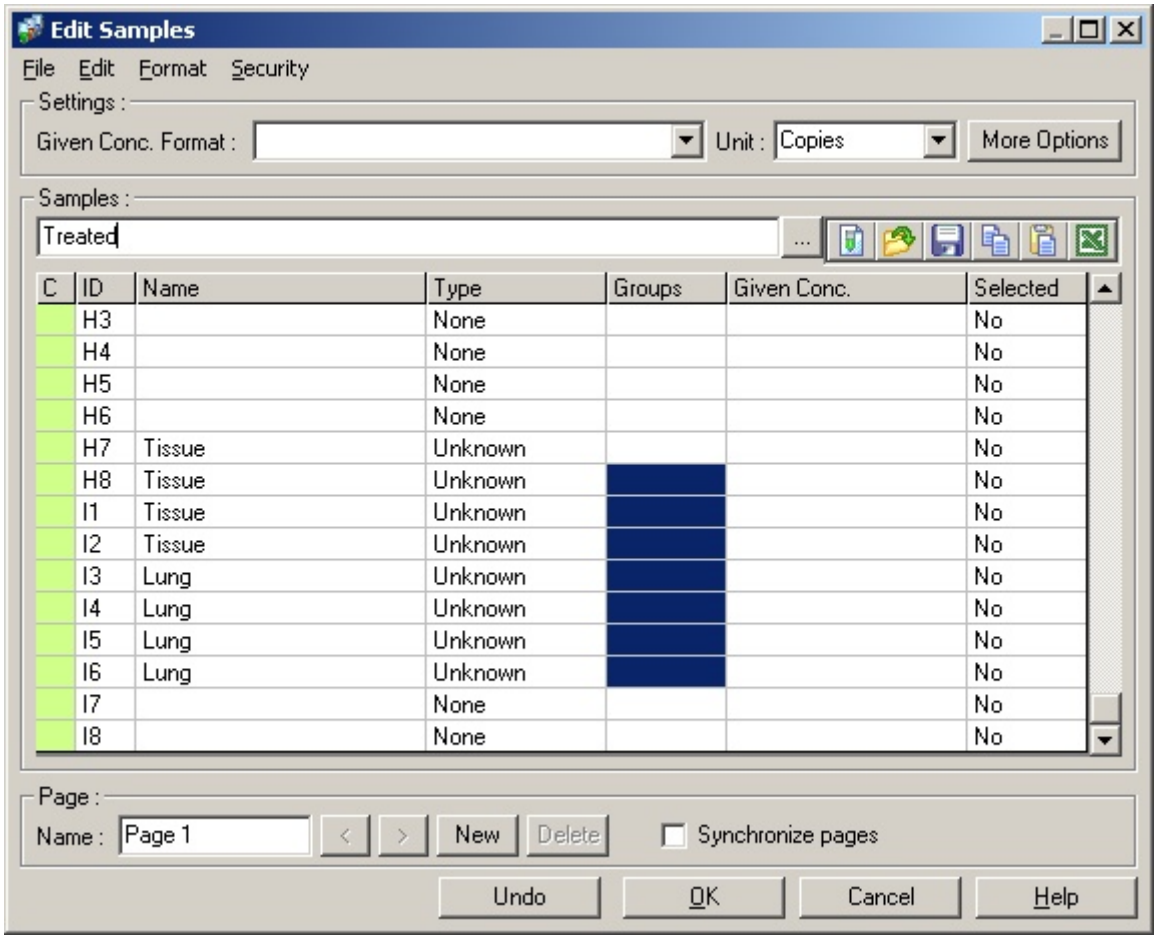


Tip: When setting up an assay, create all of the sample pages and suitabilities, then save them as a template. This reduces the amount of setup required for each run.

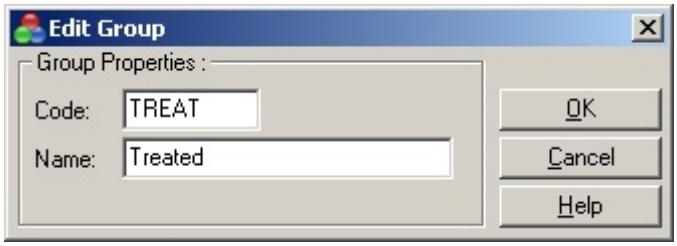
Groups

Sample groups allow you to calculate statistics for an arbitrary collection of samples. Unlike replicates, which must have identical names, samples can have any name, can be positioned anywhere in the rotor and can belong to multiple groups.

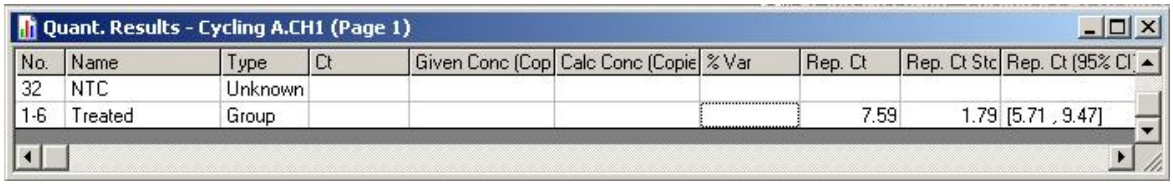
To define a group, type the full name of the group next a sample and then press ENTER:



The group editor will automatically appear:

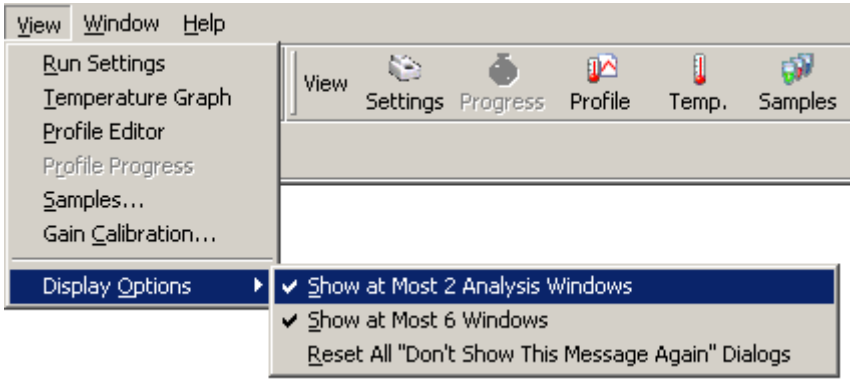


Define a suitable abbreviation, then click OK. You can now use the abbreviation to set up groups. Groups have aggregate results calculated automatically in any analysis, such as average value and 95% confidence intervals.

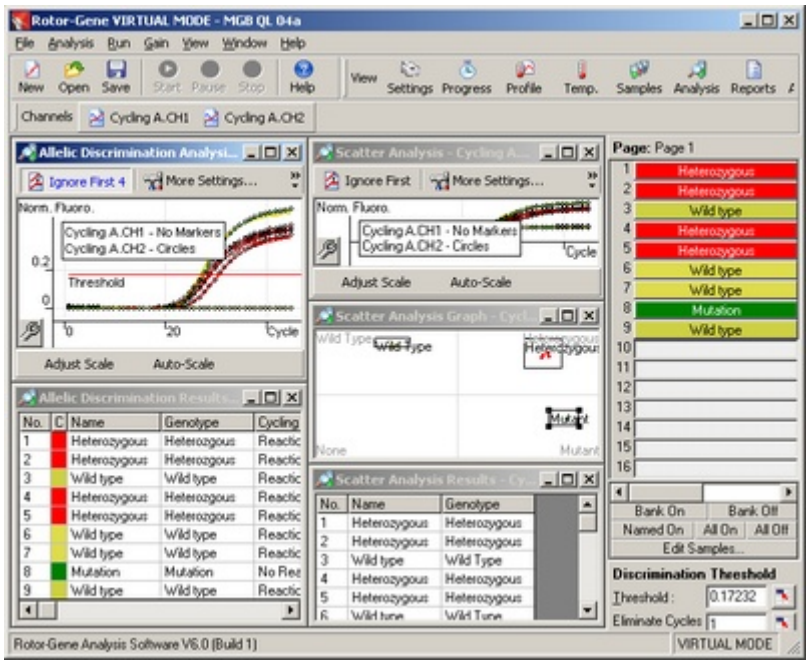


6.8.5 Display Options

The display options menu is shown below:



Show at most two analysis windows: This option ticked shows a maximum of two analysis windows at once. By performing a quantitation analysis, three windows are opened by default. If more than six or seven windows are opened at once, the overall view might not be clear. This option ticked will close the first analysis window and replace it with the last opened analysis window. If the option is unticked more than three analysis windows can be displayed. In the example below, the maximum of two windows is displayed. Opening another analysis will cause one analysis to be closed:



Show at most 6 windows: To improve readability, the software removes unused windows when new windows are opened. This option is enabled by default, as it keeps the Rotor-Gene work area clear. You may, however, need to see more than 6 windows at once, in which case you should uncheck this option.

Reset all "Don't Show This Message again" Dialogs: This window forces the software to re-

display all dialog boxes again. These include messages about suspicious settings which you may have previously set to not display again. This might be useful if a new user is now using the machine who is unfamiliar with the Rotor-Gene or the Rotor-Gene software.

6.9 Security Menu

The Rotor-Gene software contains features that enable it to operate securely in a diagnostic environment. When correctly configured, the Rotor-Gene software can ensure the following:

- Access to the Rotor-Gene equipment or the analysis software is restricted to user groups
- Modifications to run files are logged
- Unauthorized modifications are detected (signatures)
- Templates used to perform runs are logged
- Sample names are protected

Integration with Windows Security

To provide a strong level of accountability, the Rotor-Gene software does not manage security internally. Accounts, groups and passwords are all managed using the Windows inbuilt security model (Windows NT Security). Integration allows the same password that provides access to network files and programs to control Rotor-Gene access, meaning less administration. In larger organizations, for example, network administrators can easily remove access to users leaving the company due to the centralized security model.

By virtue of this, setting up the Rotor-Gene software securely primarily involves configuration of the Windows security roles according to best practices.

Prerequisites

To use security, you must be running Windows NT4 Service Pack 6, Windows 2000, or Windows XP Professional. The security features cannot be used with Windows XP Home, as it does not have the fine-grained access model used by the software. You must also have installed the software with the "Force Authentication through Windows NT Logon" option.

NOTE: The Security Menu will not appear if you are logged into a Linux Samba domain. You must have either a local logon or a Windows Server to use the described security features.

6.9.1 Configuration

This section describes how to set up the system to run the Rotor-Gene software securely.

To use the Rotor-Gene security features, you will need to have installed the software with the "Force Authentication through Windows NT Logon" option. This queries the Windows domain for your access level and credentials, and is essential for providing the accountability and security features.

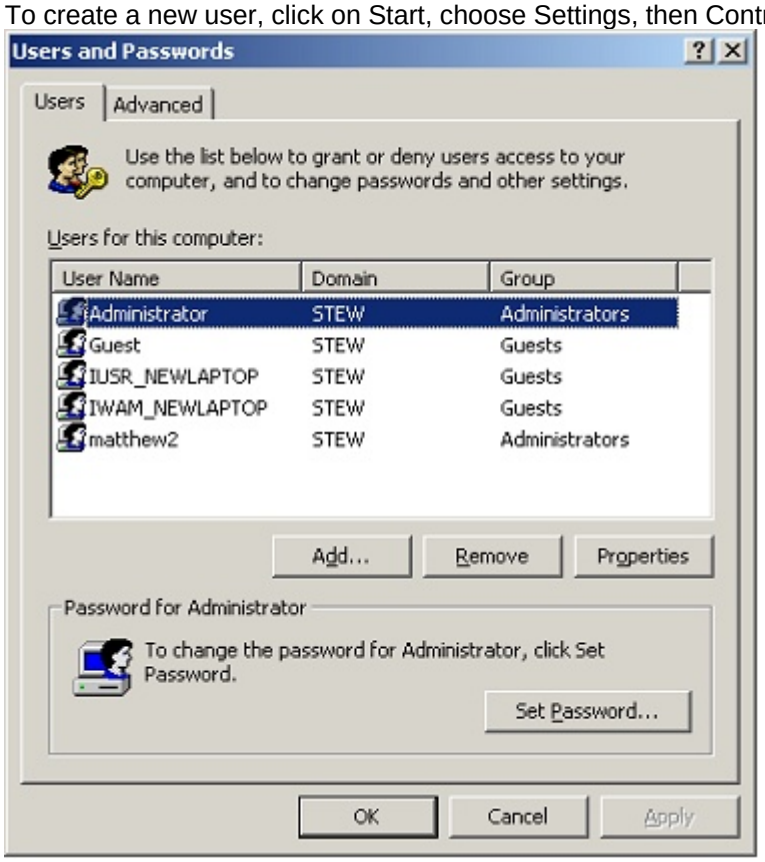
Don't Run As Administrator

Most users run their computers as 'Administrator', with no password. While this is convenient, it makes it impossible to determine who is using the computer. This eliminates accountability and prevents many of the Rotor-Gene security measures from activating. When running as Administrator, all of the software features are enabled. This ensures that users who do not need security features will not be unnecessarily surprised with restricted access to features they previously used.

Creating a New User Account

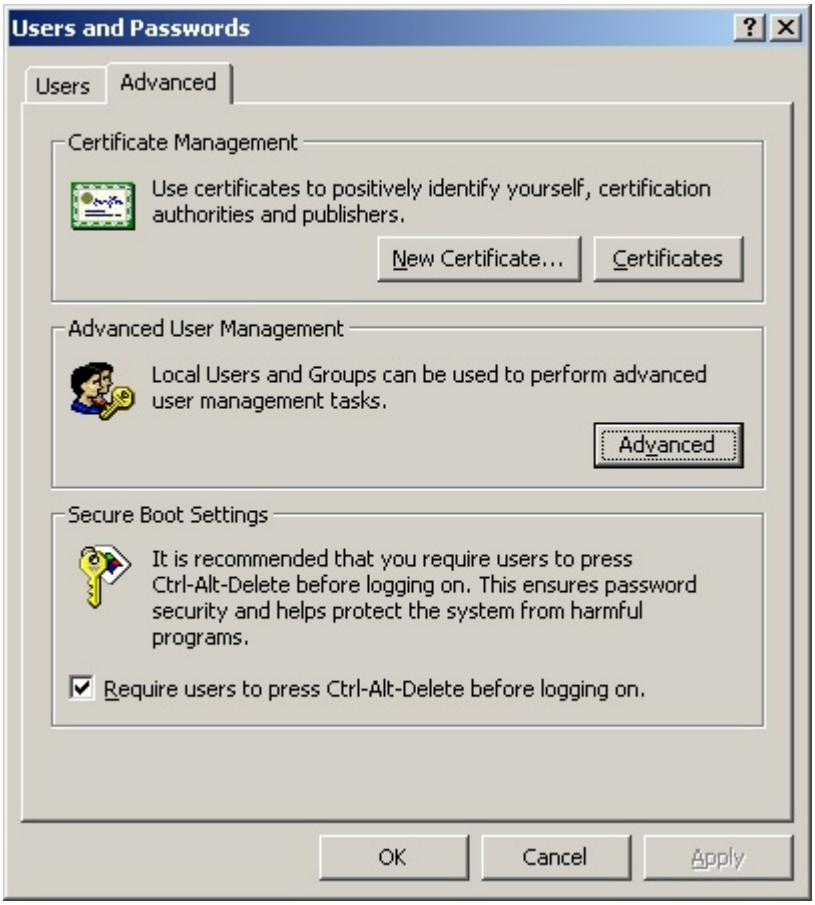
You should create user accounts for each user of the software. For each user, repeat the steps below until all accounts have been created.

To create a new user, click on Start, choose Settings, then Control Panel:

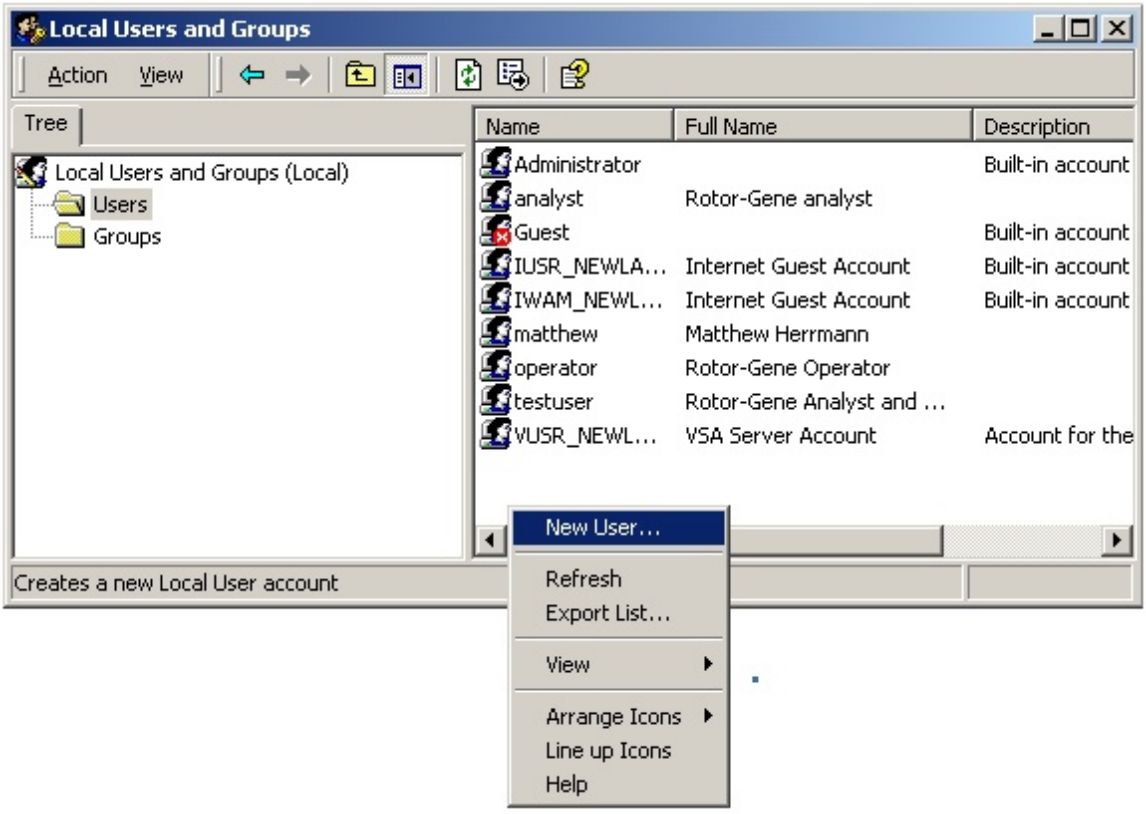


Double-click "Users and Passwords".

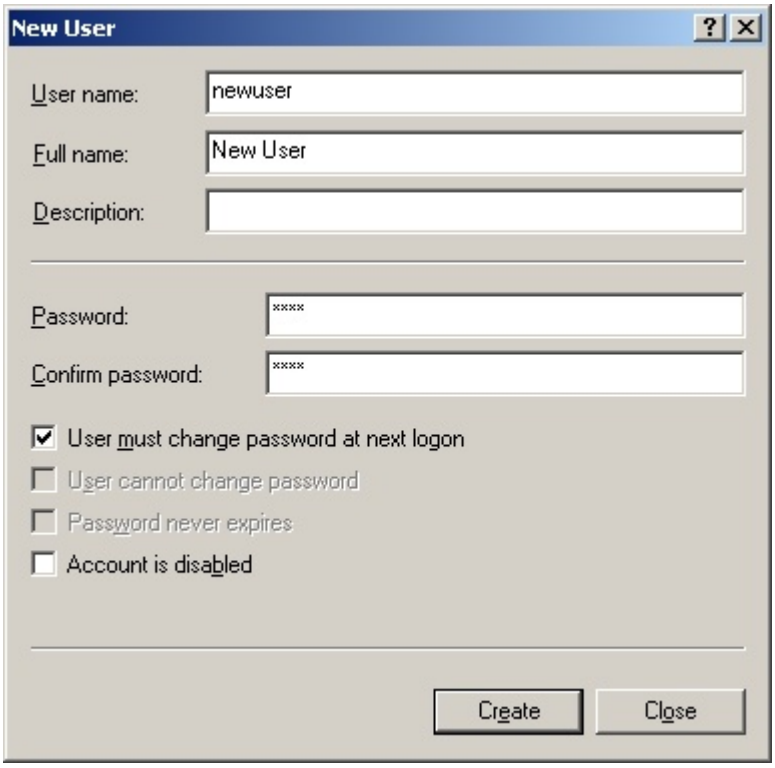
Click the Advanced tab, then click the Advanced button highlighted below:



In the window which opens, select the users folder. Right-click on the right-hand window and select "New User".



Enter a username and password. By default, the user will be created with normal access privileges. This means they will be able to run software, but not install new programs or change system settings.



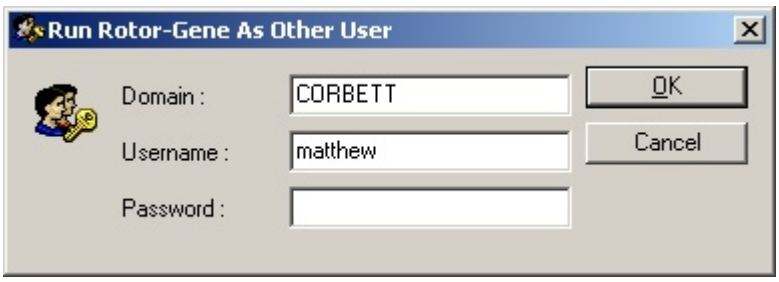
Click Create. You can now log on as this user.

Assigning Roles to Each User

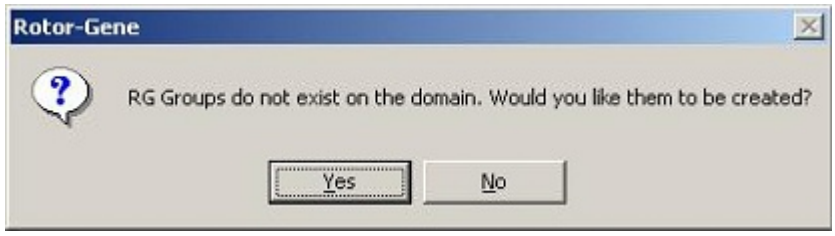
You should now assign roles to each user. Access is divided into the following areas:

- Rotor-Gene Operator – Can perform runs, but cannot generate reports or perform analysis.
- Rotor-Gene Analyst – Can analyse run data and generate reports, but cannot perform new runs.
- Rotor-Gene Operator and Analyst – Has the capabilities of both roles
- Administrator – Can unlock sample names, and perform all operations of Analysts and Operators.
- None – Access to the software is denied.

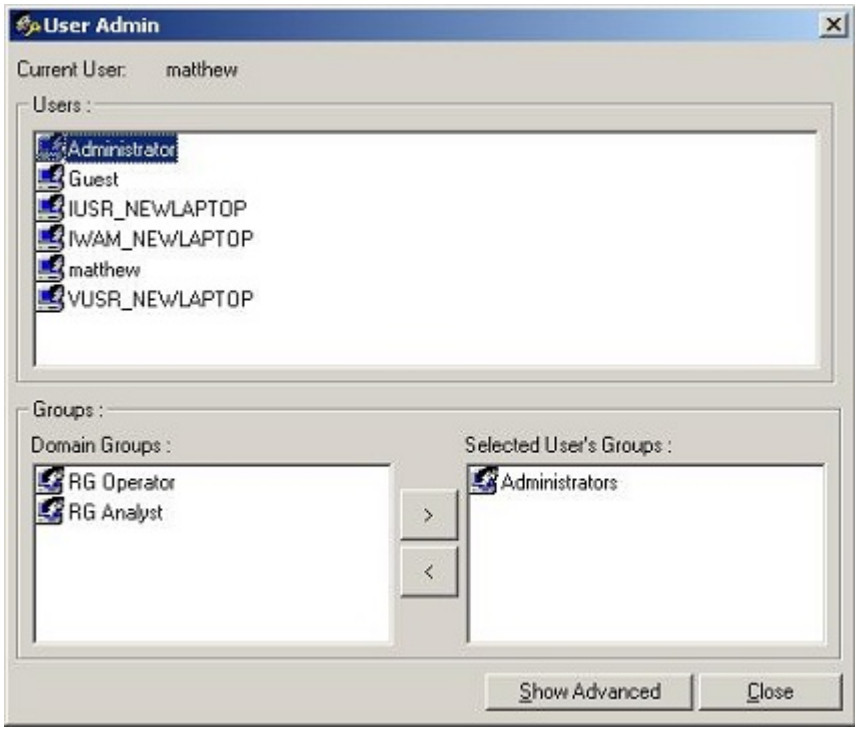
To assign roles, log in to Windows as an Administrator, or use the Rotor-Gene Logon window to open the software and log in as a single step:



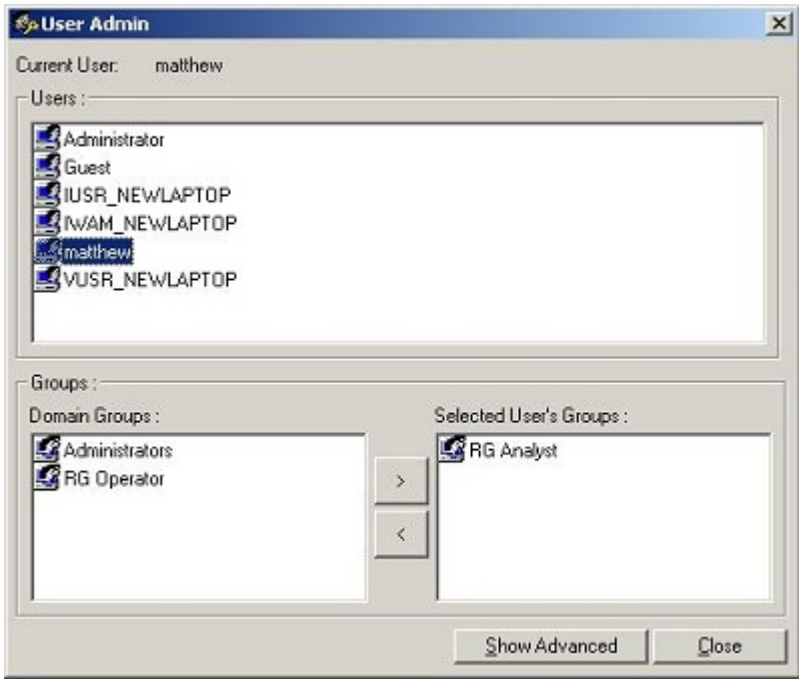
Then, once the software is open, click on the Security menu. The first time you run this, Rotor-Gene will configure a number of system groups which will control access to the software:



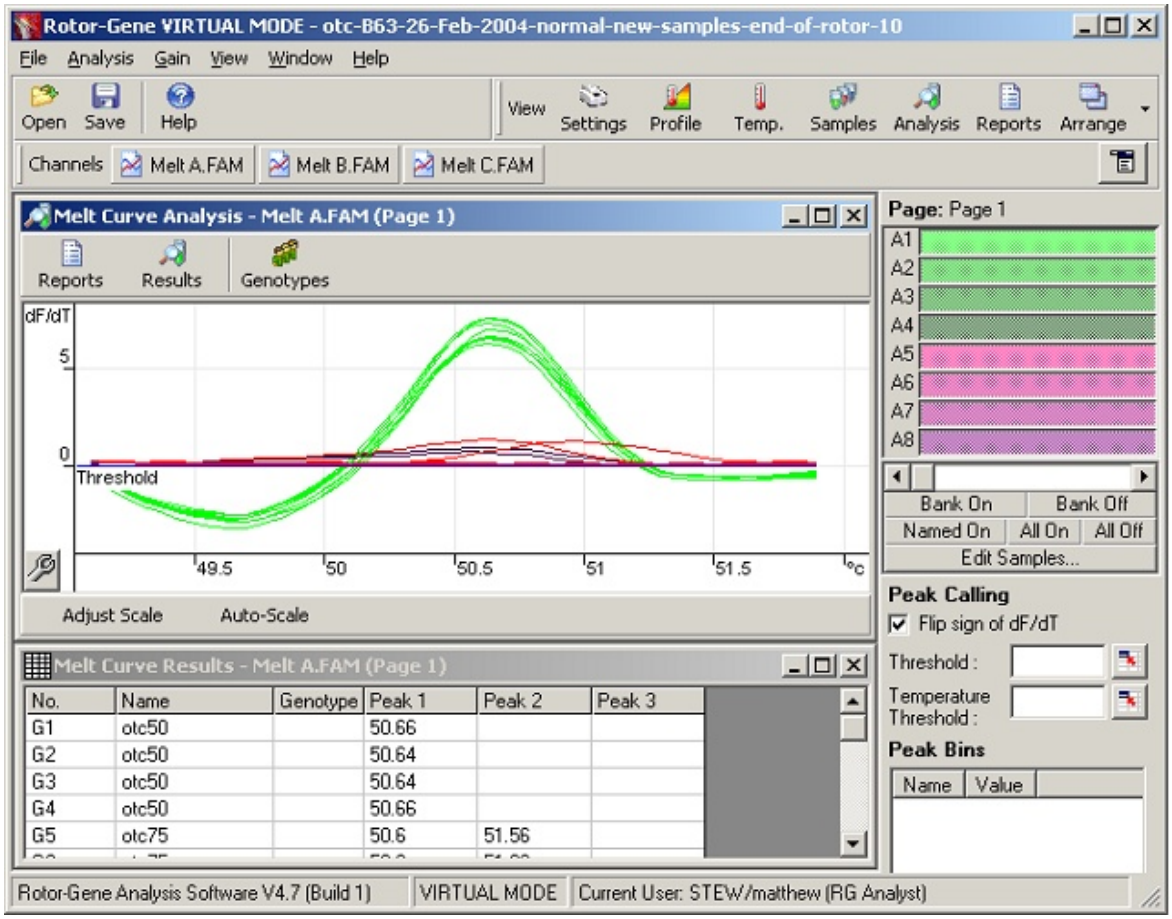
Click Yes. The main security window will then appear. In the top pane, all the users on your computer are displayed. Some accounts are used by the system, and so will be unfamiliar. The bottom pane shows the groups assigned to the user:



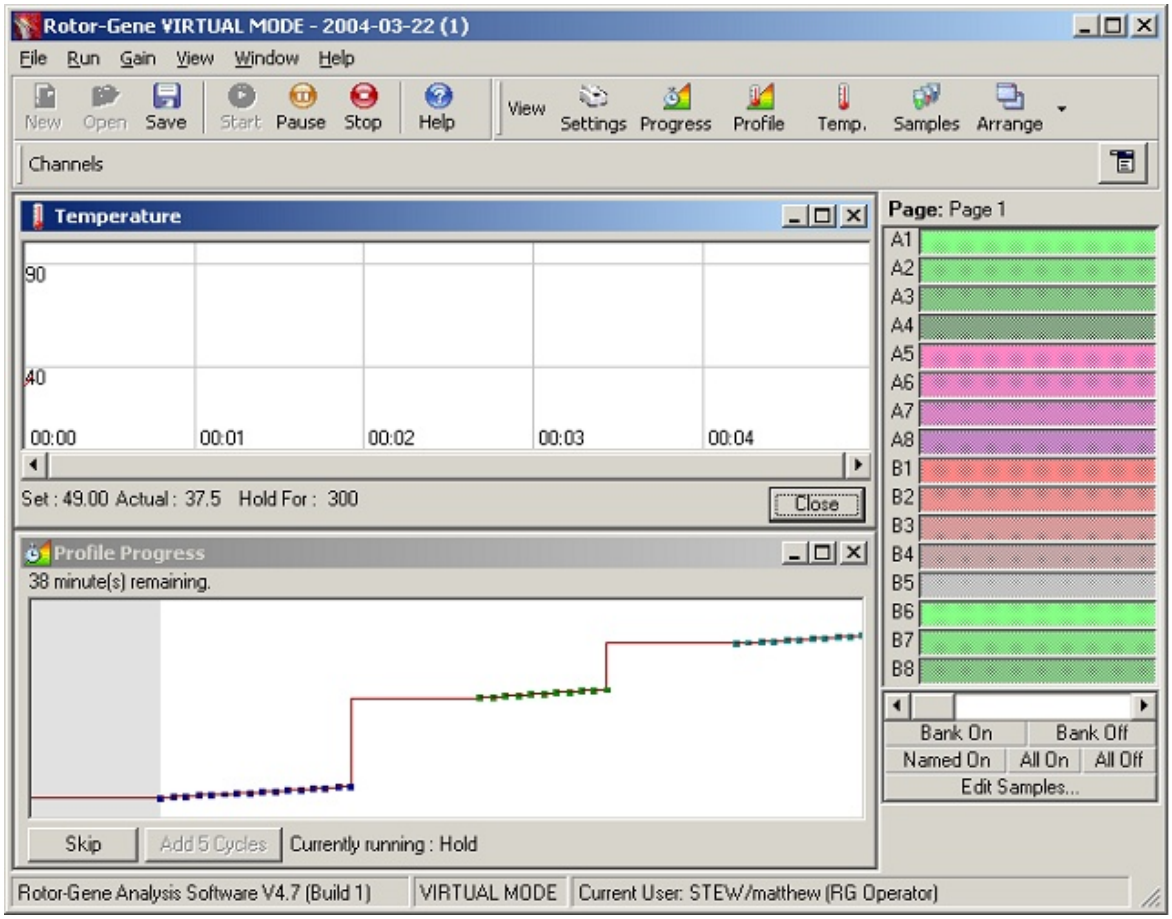
To assign a group to a user, select the user's name from the list. The bottom pane will update to reflect what roles the user currently participates in. If the user has no groups, they will not be available to launch the software. In this example, we assign the user "matthew" access under the RG Analyst group by selecting the group on the left-hand side, then clicking the [>] button. Groups can be removed by selecting them, then clicking the [<] button :



We now log in as this user. As an Analyst, the Run menu and the profile control options are unavailable. However, existing files can still be opened and analysed, as shown in the image below. The status bar indicates that the user "STEW/matthew" is an RG Analyst, thus explaining why some functionality is unavailable:



By logging in as an Administrator again, we can assign "Operator" rights to Matthew and launch the software again. This time, the Analysis and Reports options are missing, and the Run options are enabled:



The status bar indicates that the user "STEW/matthew" belongs to the "RG Operator" role.

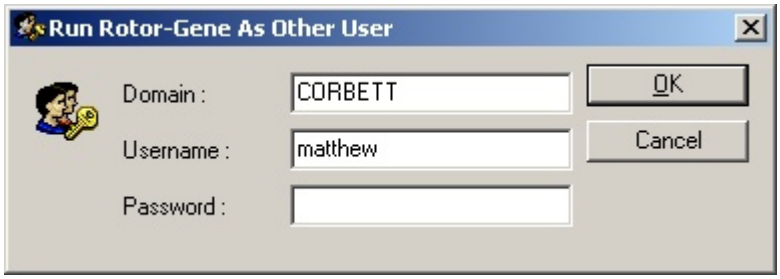
If you log in as Administrator and remove all groups from the user Matthew, the following message will appear when opening the software:

6.9.2 Running Multiple Users On Same Computer

To use the Rotor-Gene software with multiple users, you should create a user account which does not have access to the Rotor-Gene software. Log into windows using this account, so that users cannot anonymously access the Rotor-Gene instrument.

By using the Rotor-Gene 6 Login, users can open the Rotor-Gene software under their user account:

Enter your username and password in the provided box:



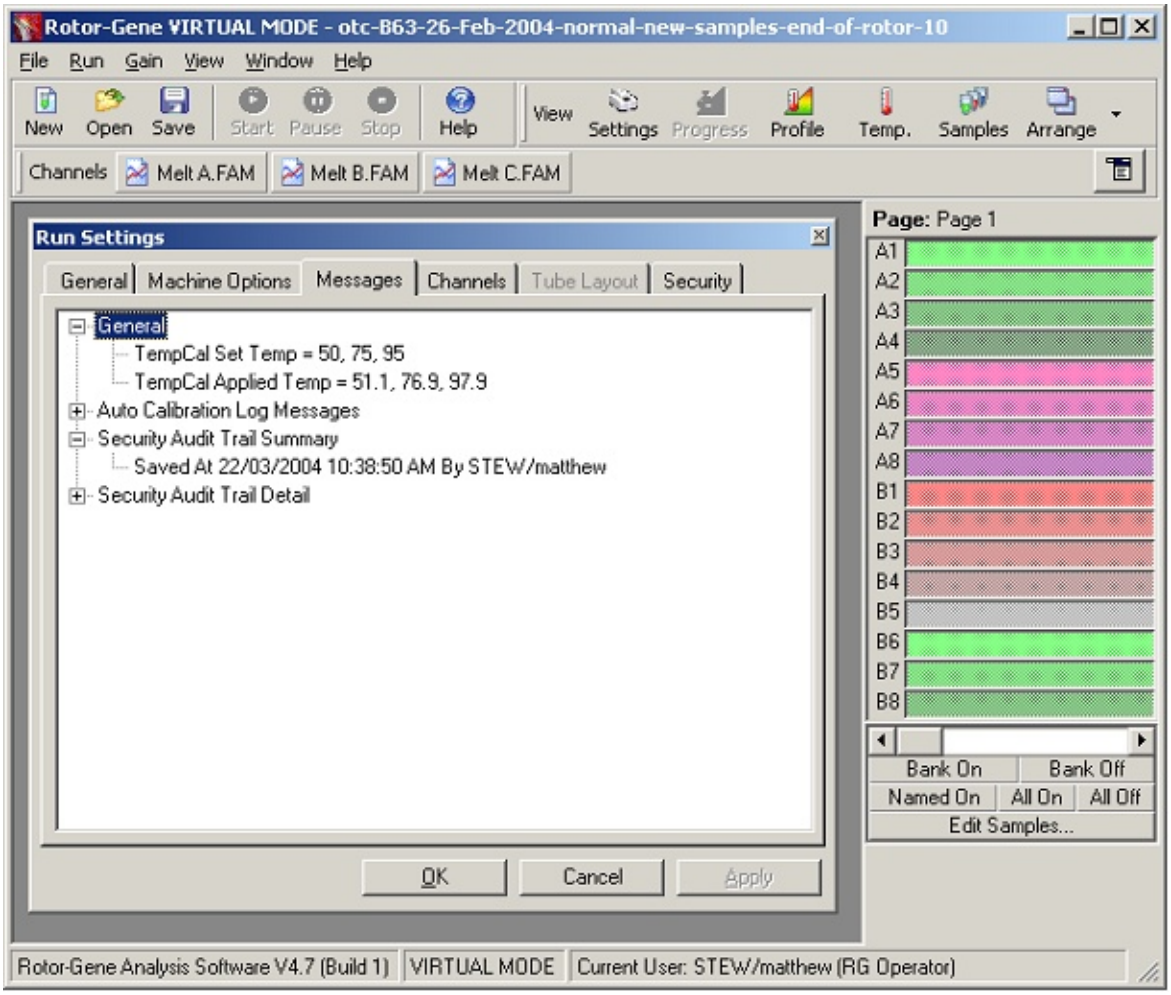
The domain is either the computer you are logging into, or the name of your local network. Consult your network administrator as to which you are logging into.

TIP: When you log into the Rotor-Gene software, all of the user files will be available for that user. You can therefore ensure that each user saves files into their own area. If set up correctly, this is the highest form of security that can be provided.

TIP: Each user should log out of the Rotor-Gene software after their run has been completed to prevent other users from performing a run in their name.

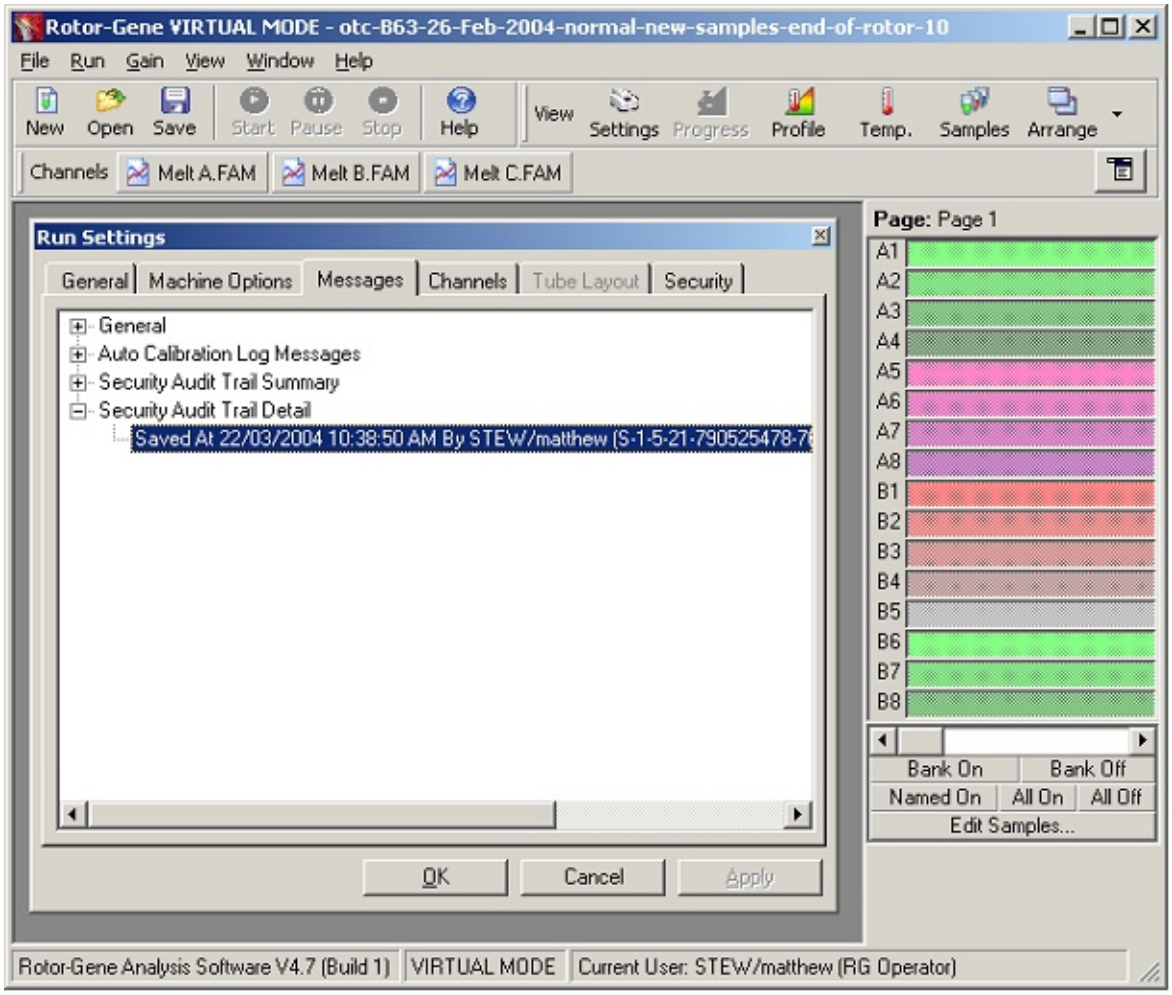
6.9.3 Audit Trails

Each time a file is saved by a user, their details are recorded in the Run Settings under the messages tab as Security Audit Trail Summary and Security Audit Trail Detail:



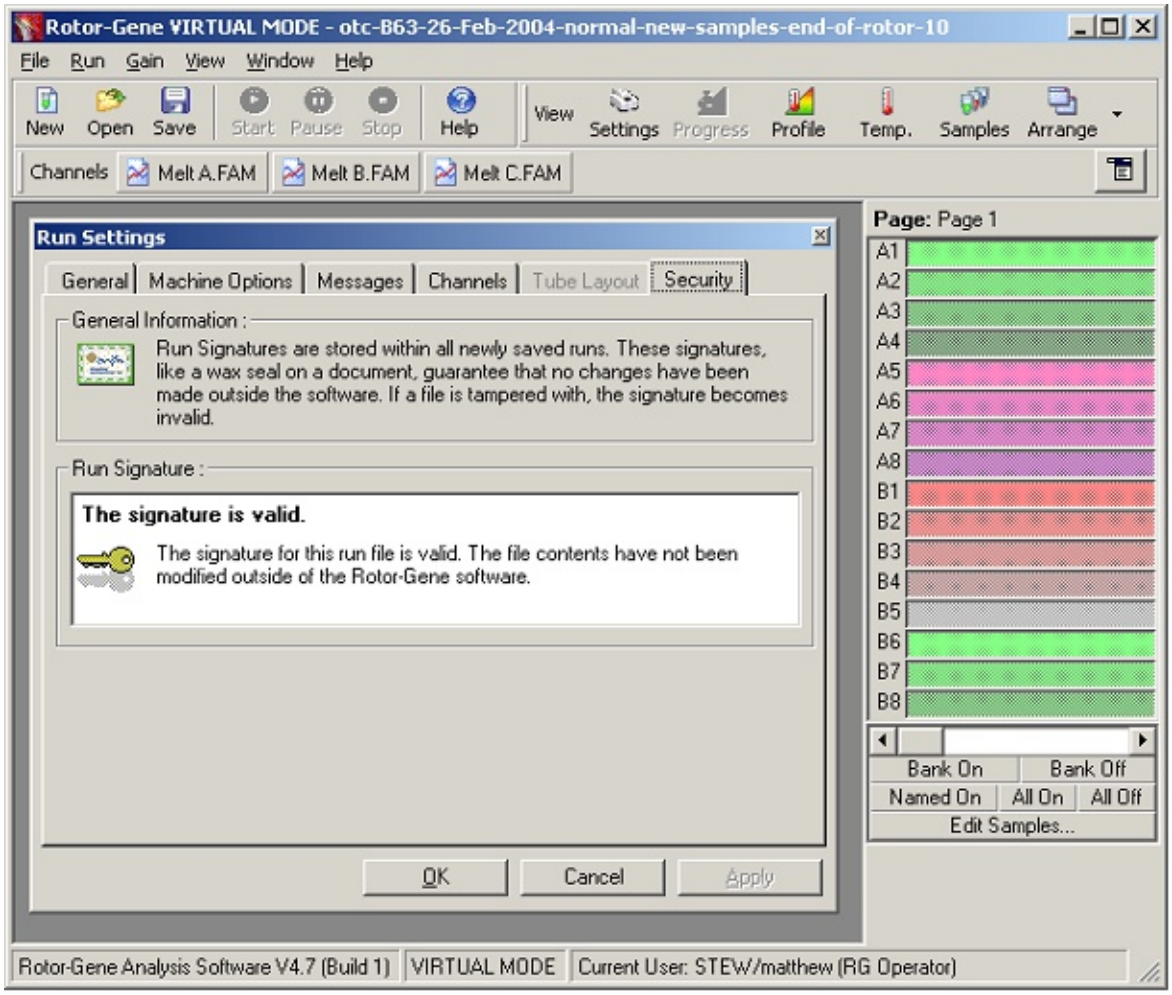
This can be used to monitor who has modified the contents of a file. The Security Audit Trail Detail contains more detail, such as the unique identifier of the user. This identifier is important to avoid a user creating an account with the same name on another computer and thereby impersonating another user. In this case, the user names will be the same, but the account id's will be different.

The identifier for the account STEW/matthew, S-1-5-21-790525478-... is shown in the details:

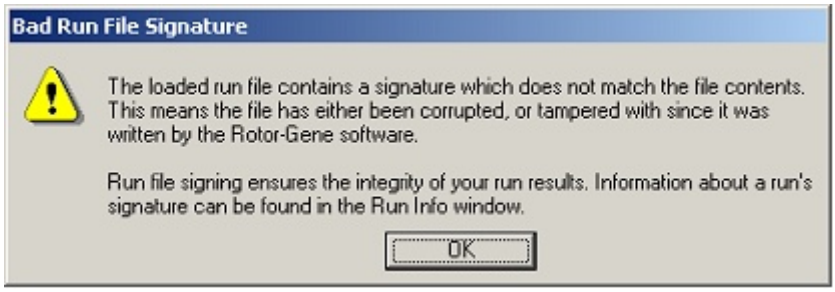


6.9.4 Run Signatures

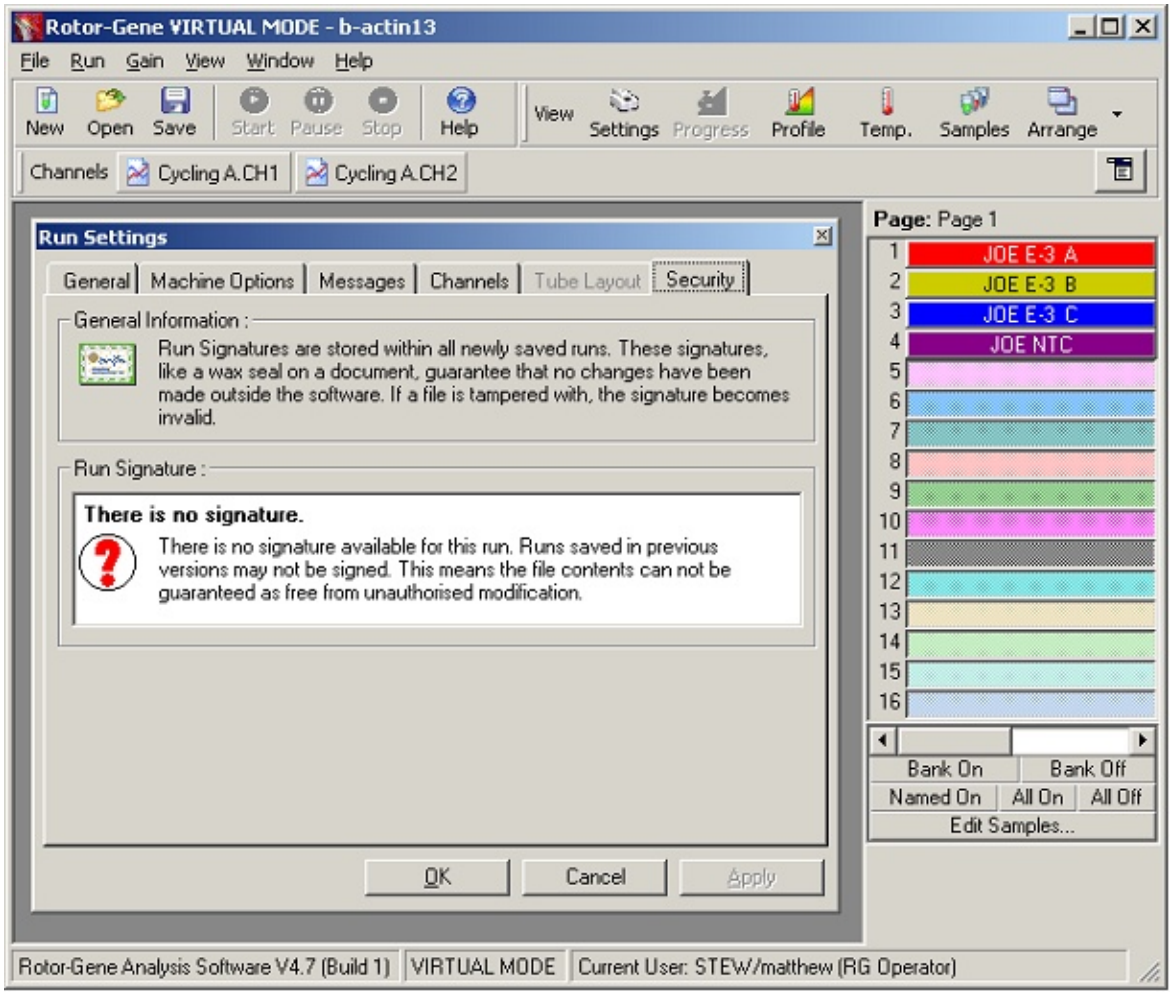
All of the audit trail is stored within the Rotor-Gene run file. Conceivably, this file could be modified by a malicious user to remove any trace of their actions. The best defence against such damage is to keep the files in a safe location. This can be done by setting up Windows accounts to only have access to certain files. However, when files are being stored in a shared area, Run Signatures can provide an extra degree of security against such modification. Here is a screenshot of the Security tab in Run Settings, with a signed run file:



The Run Signature is a long word generated each time the file is saved which is linked to the contents of the file. For example, the signature for this file is "517587770f3e2172ef9cc9bd0c36c081". If now opened in notepad, and the run date is changed 3 days earlier, the following message appears when the file is reopened:



Run file signatures were introduced in version 5.0 of the Rotor-Gene software. Old run files will therefore, not contain signatures. You should treat with suspicion, however, any new runs that have been performed which do not contain a signature:

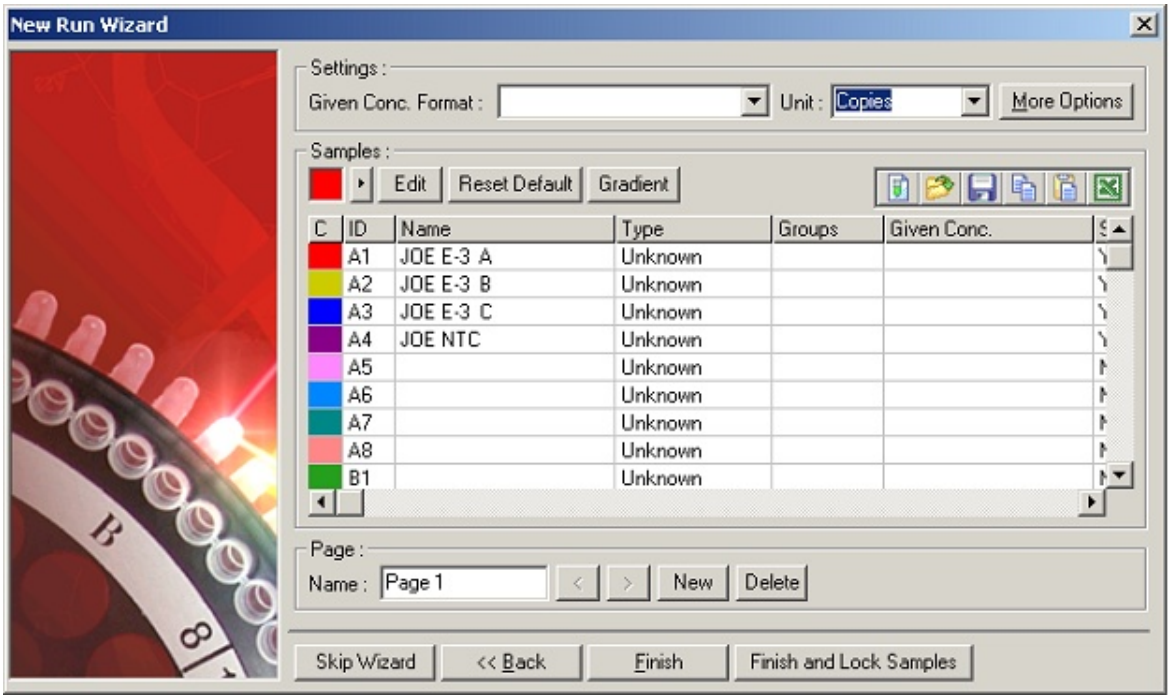


6.9.5 Sample Locking

In a diagnostic environment, it is important to ensure that sample names are not accidentally or intentionally changed once an operator has started a run. To allow for this, the Rotor-Gene software provides a sample locking mechanism. The sample names can be locked by any user, but can only be unlocked by an Administrator. Since most users run their computers in Administrator mode, this option will be of limited use unless the computer has been configured securely as described in the previous sections.

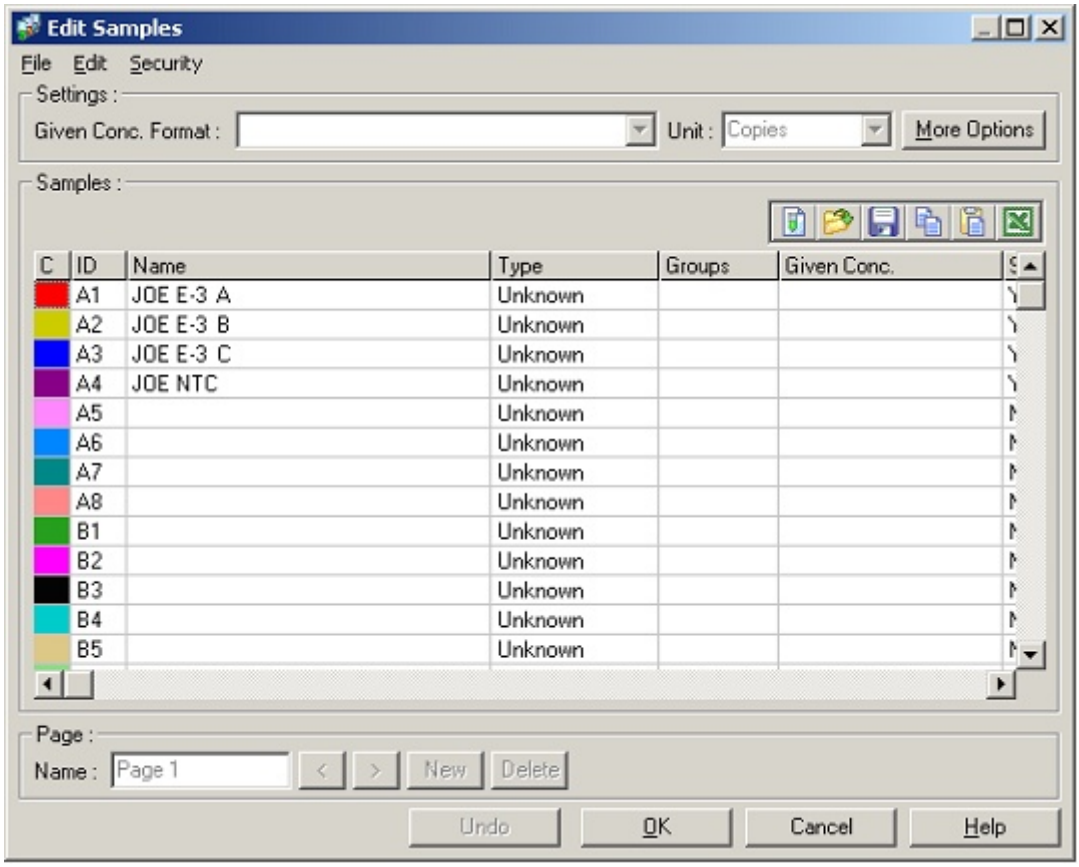
Tip: If you intend to lock samples, do not run the software as Administrator. Create an account with RG Operator and RG Analyst access, and do not make the Administrator password publicly available. Users will then require your authorisation to unlock files that contained mistakes.

Samples can be locked before starting a run when using the Advanced Wizard, by clicking "Finish and Lock Samples":

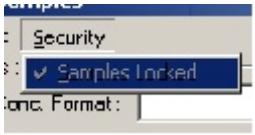


The following warning will appear. Click Yes to confirm.

Upon editing the samples in the Sample Editor, all editing functionality will be disabled:



Sample Locking can also be toggled inside the Sample Editor. However, unless you are an administrator, the Sample Locking menu item cannot be toggled once it has been set:



As before, it is possible for users to modify the run file and remove the locking flag. However, as an unauthorized change to the file, this will again break the run file signature, alerting you to the violation.

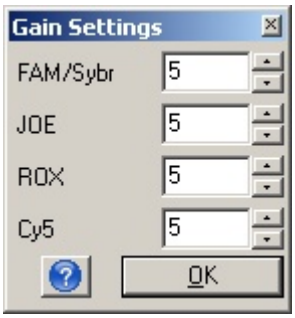
6.9.6 Locked Templates

In a diagnostic environment, it may be a requirement that all runs be performed by launching a specific template file. This template may be stored on a network drive where users cannot modify data. Users still have the possibility of running their own profiles, however. To account for this, the Rotor-Gene software stores the name of the template file that was run, accessible via the Run Settings menu in "Other Run Information":

It is not currently possible to create read-only template files through the Rotor-Gene software interface. However, templates, such as those used for the Temperature Verification Rotor can be locked by the development team by placing a request on our support address support@corbettresearch.com. Functionality to lock templates within the software is scheduled for a future release.

6.10 Gain Menu

Here, you can view the **Gain Settings** for the current run and modify them if the run has not yet been started. Use the **Up/Down** arrows next to each text field to modify the fields without needing to enter in a value with the keyboard. Click OK when you have finished.



Sets the Gain of the specified channel before running a program. Gain settings will be retained from the last run.

Change gain during the first ten cycles: This new software version also offers the possibility to change the gain during the initial cycles. A red line will be drawn in the appropriate channel showing where the gain has been changed. The cycles before the gain has been changed will be excluded from analysis.

6.11 Window Menu

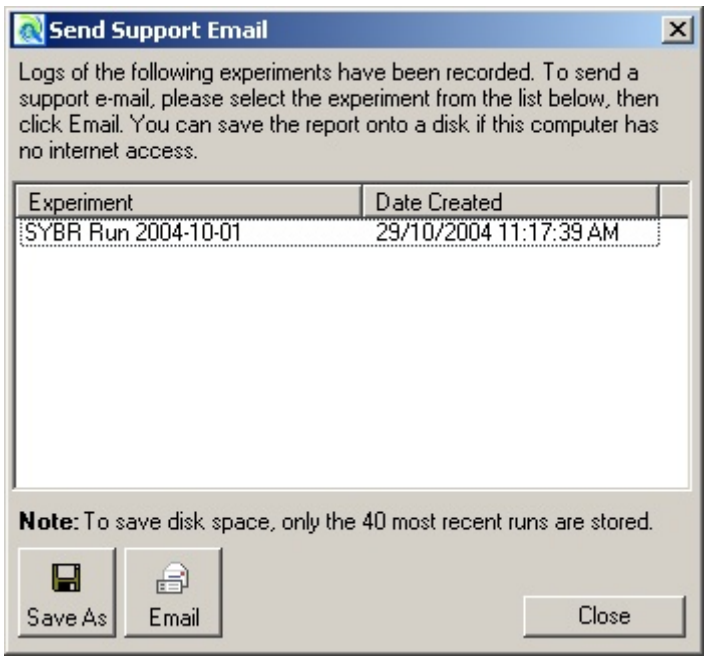
This menu allows you to tile the window either vertically, horizontally or arrange windows in a cascade. A further option of tiling windows is found under Arrange in the tool menu.

6.12 Help Menu

The **Contents** menu brings up the help menu. **What's New** gives a brief overview of new features added to the previous software release. Selecting **Website** will open the site in a new browser window. **About Rotor-Gene** gives miscellaneous information about the connected machine, the serial number of the Rotor-Gene and the software version.

6.12.1 Send Support E-Mail

The **Send Support Email** option in the Help menu allows you to send a support email to Corbett Research which contains all relevant information from a run. The **Save As option** will save all the information to a file which you can copy onto a floppy disk or across a network if you do not have access to e-mail on the Rotor-Gene computer. Technical support is available via phone on +612 9736 1320 or email at support@corbettresearch.com.



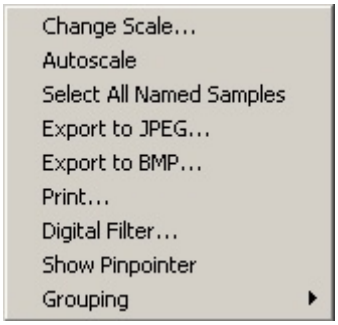
7 General Functions Used Over Several Windows

7.1 Opening A Second Run

While performing a run, it is possible to open and analyse runs which were performed earlier. Note that several functions like **New** or **Start Run** are not activated in this second window. Even closing the first window will not bring up these functions. A new run can only be started from the first window, once the first run has finished. If a run must be started from the second window, it needs to be closed and reopened to access the appropriate functions.

7.2 Spanner Icon

Clicking the spanner icon at the bottom left-hand corner of each window brings up a number of miscellaneous options. These tools can also be accessed using the right mouse button on the graph directly.

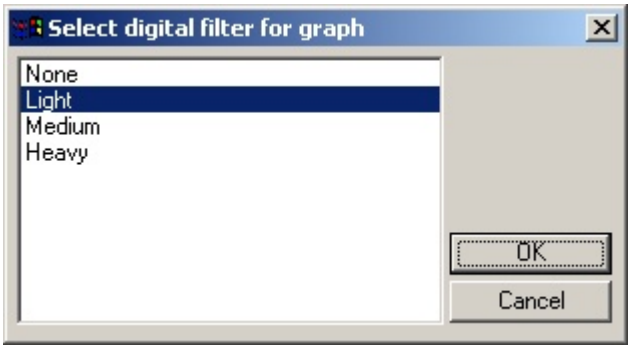


Scaling: See above.

Export to JPEG... Saves the graph in the JPEG image format and **Export to BMP...** saves the graph in Windows Bitmap format.

Print: This function allows for the current graphic screen to be printed.

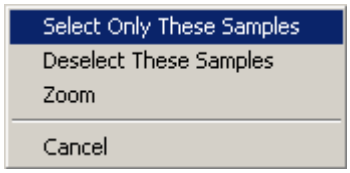
Digital Filter: Modifies the currently selected digital filter on the graph. The digital filter smoothes data using a sliding window of points. To open the window, click the right mouse button over a window containing data.



Show Pinpointer: Opens a window with exact coordinates of the current cursor position.

Grouping: Visually groups samples which have been given identical names. This can be useful on full rotor runs. Toggling this option has no impact on calculated values.

Dragging with the Mouse: Once a region has been dragged with the mouse, the following options are available:



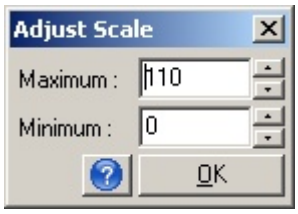
Select Only These Samples: Samples not contained in the highlighted region will be deselected.

Deselect These Samples: All samples in the selected region will be deselected.

Zoom: Zooms to the highlighted region of the graph. Clicking **Default Scale** will zoom out again.

7.3 Scaling Options

Adjust Scale will bring up a window in which you can manually enter a scale, or interactively select one. To chose this option simply press the right mouse button over the appropriate screen.



Auto-Scale attempts to fit the scale to the maximum and minimum readings in the data.

Default will reset the scale to display from 0-100 fluorescence units.

7.4 Abbreviations

BHQ	Black Hole Quencher
Comp. Quantitation	Comparative Quantitation
LCD	Liquid crystal display
LED	Light Emitting Diode
PMP	Photomultiplier
Quant. Settings	Quantitate Settings
.REA	Rotor-Gene Archive File
.REX	Rotor-Gene Run File
.SMP	Sample File
Std. Curve	Standard Curve

8 Hardware Information

8.1 Filter Specifications

SYBR-Green I is detected on the preset FAM/SYBR channel, which has a channel configuration of 470nm/585hp.

Dual Labeled Probes

Fluorophores	Excitation Maxima	Emission Maxima	Recommended Filter Setup
5-FAM, 6-FAM:	495	520	FAM/SYBR Channel
JOE	520	548	JOE Channel
TET	521	536	JOE Channel
VIC	524	555	JOE Channel
Yakima Yellow	525	548	JOE Channel
HEX	535	556	JOE Channel
Cy3	550	570	470/585 hp or 530/585 hp
TAMRA	555	576	530/585 hp
ROX	575	602	ROX Channel
Cy3.5	581	596	ROX Channel
Texas Red	596	620	ROX or Cy5 Channel
Red 640	625	640	Cy5 Channel
Cy5	649	670	Cy5 Channel
LC 705	685	705	Cy5 Channel

For multiplex reactions we recommend using FAM, JOE, ROX and Cy5, since these fluorophores give a cross talk less then 1% after optimization.

Quenchers to be used for the above dyes:

BHQ-1: 480 – 580
BHQ-2: 550 – 650
BHQ-3: 620 – 730
TAMRA for FAM or JOE (VIC)

FRET Analysis

FAM and JOE, for example, should be detected on the above mentioned channels (quenched FRET). If Cy5 is to be detected (normal FRET), the filter combination should be configured as follows:

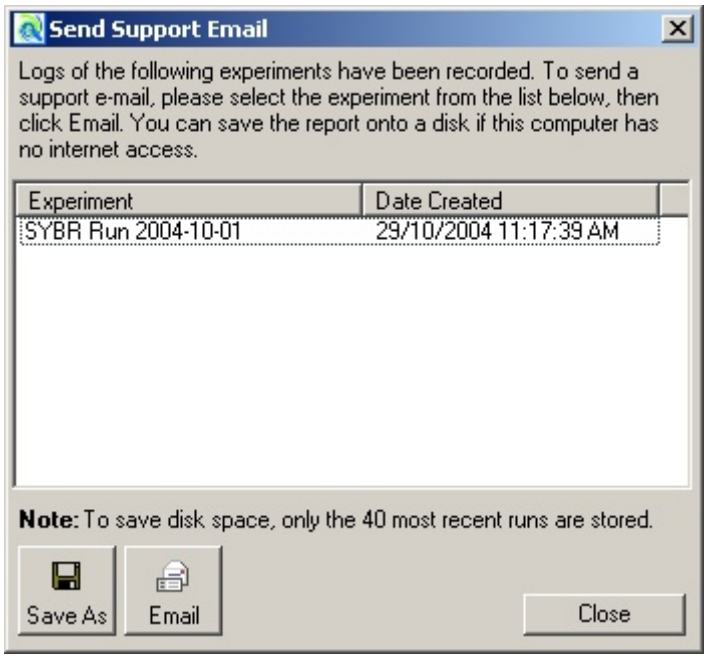
FAM-Cy5, FAM-LC 640, FAM-LC 705	470/610 hp	FAM-BHQ1:	FAM/SYBR Channel
JOE-Cy5	530/610 hp	JOE-BHQ1:	JOE-Channel

9 Troubleshooting

9.1 Log Archives

The software keeps an unmodified record of each run, along with diagnostic information in its Log Archive repository. By using the Help, Send Support Email option, you can send an email with all the diagnostic information the Corbett Research support technicians require:

See the section Send Support Email for more information.



9.2 Troubleshooting Run Files

To troubleshoot runs which did not perform as expected, enter the Profile Editor and ask the following questions:

9.2.1 Initial Denature Step

Is the initial hold temperature and time for the Taq Polymerase used appropriate? Check the manufacture's description of the enzyme. The recommended temperature is 95°C and the times can vary from 2 min to 15 min, depending on the enzyme used.

9.2.2 Cycling Profile

Have 50 µl volumes been run on the Rotor-Gene?
The Rotor-Gene is calibrated for 20 – 25 µl, in which case we recommend a denaturation time during cycling of 15 seconds. Some protocols specify 50 µl reactions in which case we recommend increasing the denaturation time during cycling to 30 seconds.

Is the denature temperature appropriate? Most double stranded DNA has melted at 95°C. In some cases temperatures from 90°C to 94°C may not denature the DNA properly. Due to partially denatured DNA, the accessibility of primers and probes could be reduced and could therefore reduce the reaction efficiency of the assay.

Is the denature time appropriate? 15 to 20 sec is usually enough to denature the amplicon, however, longer products might need up to 30 sec. A denature time of 60 sec is usually not required.

Is the annealing temperature and time appropriate? Check the Tm's of the primers and probes. The annealing time for SYBR-Green I is around 20 to 35 sec. Dual labeled probes are often run as a two step profile where annealing and extension steps are combined. In this case the annealing/extension step is between 45 and 60 sec and the temperature is usually 60°C. For FRET probes the annealing step is between 20 and 30 sec.

Is the extension temperature and time appropriate? Check the recommendations of the manufacturer's Taq Polymerase. For a product less than 300 bp the recommended time is between 15 and 30 sec.

At what step have data been acquired? For SYBR-Green I data should be acquired at the end of the extension step. Dual labeled probes are often run as two step assays, with data acquisition on the lower temperature step. For FRET assays, data should be acquired at the annealing step. If in doubt about the appropriate data acquisition point, data can be acquired at several steps for comparison. If no raw data can been seen on the screen at all, go to edit profile and check if data was acquired at least at one point.

9.2.3 Melt Curve Analysis

Has a hold step at the same temperature as the beginning of the melt been inserted before starting the melt curve? If no hold step has been used before running a melt curve, the results in the melt curve analysis could be a steep increase in the first few cycles making the actual melt peak hardly visible.

For SYBR-Green I melt curves; was the melt curve run to 99°C? Some amplicons melt higher than 90°C. The expected melt peak might not occur, due to the fact that DNA has not melted.

9.2.4 Gain Settings

Have the appropriate gain settings been chosen? In some cases it has been observed that curves in the raw data went off scale resulting in a straight line at a fluorescence of 100. Although quantitation of most data can still be performed, the gain should be reduced to a setting, where the raw data does not go off scale.

In some cases it has been observed that fluorescence signal is off scale even at the first cycle, thus appearing as if no data is being acquired. This indicates that the gain has been set too high.

If a straight line at a fluorescence of 100 at the beginning of a melt curve is observed, the melt curve should be re-run using a lower gain. There is no need to repeat the amplification as SYBR-Green I or FRET samples can be reused to a certain extent for melt analysis.

Have the appropriate Quantitation Settings been chosen? In some cases it has been observed that the raw data show a perfect amplification but no data can be seen in the quantitation screen. Setting the NTC Threshold to zero, and disabling the Reaction Efficiency Thresholds will show all data. The Quantitation Settings can be used to exclude small changes of fluorescence, due to probe degradations or other probe related effects, which do not show true amplification but rather a steadily increasing line.

If the quantitation curves can be excluded with minimal Quantitation Settings, the gain for the next run should be increased. Note: The gain setting has no influence on the actual data, the expected fold increase will be similar.

9.2.5 General Setup Conditions

Has the appropriate setup been chosen for the Rotor-Gene? To check, go to file menu then to setup and tick the appropriate boxes. If in doubt about the meaning of any other the settings, or if setup is not accessible, please contact the distributor.

Has the appropriate rotor for the run been chosen? At the beginning of the run wizard, ensure that the selected rotor corresponds to the one installed in the Rotor-Gene instrument. Selecting a mismatched rotor setup will result in the loss of the data from every second sample (when running a 72-well rotor but having selected the 36-well setup) or incorrectly labelled positions (when running a 36-well rotor with the software configured for the 72-well rotor).

What version of the software is used on the Rotor-Gene? The software development for the Rotor-Gene is ongoing. Check our website for additional information. Make sure that a recent version is uploaded and used.

9.2.6 Run Settings

If samples are not detected check also the channel setup. Make sure data has been acquired with the appropriate excitation and detection filters. This is particularly important when user defined filters have been set up.

What if all the above mentioned conditions were right, but the assay did not look as expected or did not work at all? In the restricted area of our homepage there is a file called "real-time summary". Steps are suggested on how to optimize SYBR-Green I, dual labeled probes and FRET reactions. In general, the expected fold increase of a SYBR-Green I reaction is between 5 and 50 times and a dual labeled reaction between 2 and 10 times.

9.3 Regional Settings In Windows 98

On some computers, the regional settings are configured in a contradictory manner which causes a conflict within the Rotor-Gene system. The problem occurs when different decimal settings are used for currencies and numbers. For example, if numbers are displayed in a "123 456,789" format, but currencies are shown as "$299,192.20", then there is a conflict, since the number "20,123" is ambiguous. On Windows 98 machines, this prevents the Rotor-Gene software from correctly interpreting numbers. This problem does not occur on Windows 2000.

To correct this setting, please follow the following steps. The names of these options may be different in your system's language.

- Click on the Start Menu.
- Click on Settings.
- Click on Control Panel.
- Double-Click on Regional Settings.
- Double-Click on Regional Options.
- Click on the Numbers tab.
- Note down which is the Decimal Symbol, and which is used as the Digit Grouping Symbol. On a German system, for example, the comma (,) will be used for Decimals, and the period (.) is used as a Digit Grouping Symbol.
- Click on the Currency tab.
- Change the Decimal Symbol and the Digit Grouping Symbol to be the same as on the Numbers tab.
- Click OK.
- Re-run the Rotor-Gene software.

10 Mathematical Appendix

This section describes the mathematical techniques used in more detail.

Quantitation

Calculated concentrations are obtained from a simple linear regression model, with the known values the log concentrations (x) and the experimental values the C_T values (y).

The log concentrations and C_T values of the standards are used to construct a model in the form:

y = mx + b

Confidence Intervals for Calculated Concentrations

We use the following confidence interval 100(1-α)% for an estimate of a new observation x0 from the standard curve.:

$$\frac{Y_0 - \hat{\beta}_0}{\hat{\beta}_1} \pm \frac{S}{\hat{\beta}_1}(1 + \frac{1}{n} + \frac{(x_0 - \bar{x})^2}{S_{xx}})^{\frac{1}{2}} t_{n-2,\alpha/2}$$

This is the confidence interval for the concentration of a single unknown.

Suppose now we have k further observations at x=x0 and we denote their average by $\bar{Y}_0$. Then,

$$\bar{Y}_0 \sim N(\beta_0 + \beta_1 x_0, \frac{\sigma^2}{k})$$

and arguments similar to above give

$$\frac{Y_0 - \hat{\beta}_0}{\hat{\beta}_1} \pm \frac{S}{\hat{\beta}_1}(\frac{1}{k} + \frac{1}{n} + \frac{(x_0 - \bar{x})^2}{S_{xx}})^{\frac{1}{2}} t_{n-2,\alpha/2}$$

This formula determines how confidence intervals for concentrations of replicate unknowns are determined.

For estimation of standards, a tighter confidence interval can be obtained :

$$\frac{Y_0 - \hat{\beta}_0}{\hat{\beta}_1} \pm \frac{S}{\hat{\beta}_1}(\frac{1}{n} + \frac{(x_0 - \bar{x})^2}{S_{xx}})^{\frac{1}{2}} t_{n-2,\alpha/2}$$

The implication of this formula is that adding replicates to an standard individual concentration reduces the width of the interval for all estimates, as n is increased. Adding a large number of replicates to an Unknown reduces its uncertainty to that of a single standard. The extra replicates reduce the uncertainty due to the unknown not forming part of the linear model.

Confidence Intervals for C_T Values

We assume that error in replicate C_T values is linear and normally distributed.

We therefore use the One-Sample t Confidence Interval. Let μ be the mean value for a replicate's C_T values $(x_0 \ldots x_{n-1})$. Then, a 100(1-α)% confidence interval for a C_T value μ is:

$$\left(\bar{x} - t_{\alpha/2,n-1} \cdot \frac{s}{\sqrt{n}},\ \bar{x} + t_{\alpha/2,n-1} \cdot \frac{s}{\sqrt{n}}\right)$$