bms
bio molecular systems

mic
magnetic induction cycler

User Manual

Verison 1.2

Contents

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The Mic instrument has been designed and is intended for Research Use Only.

Some of the polymerase chain reaction methods involve patented technology. Use of the Mic instrument for specific intended use in PCR may require the user to obtain rights from third parties. It is the sole responsibility of the user to obtain all rights necessary to allow for the intended use.

Technical Support

Bio Molecular Systems provides customer support for all technical and service issues related to the Mic instrument. For technical support, please contact our support staff via:

Address:	Suite 504, 24 – 30 Springfield Ave, Potts Point NSW 2011, AUSTRALIA
Phone:	+61 (02) 9332 1694 (Hours of operation are 9:00 – 17:00 AEST)
Email:	support@biomolecularsystems.com
Web:	www.biomolecularsystems.com

Intended use of the Mic Instrument

The Mic instrument is intended to be used to perform qPCR and melting, for molecular biology research applications that include medical, agricultural, forensic science and basic life science.

The Mic instrument is intended for use by laboratory technicians and physicians trained in molecular biology.

Mic is intended for research use only.

Safety Information

Before using the instrument, it is important to read this user manual to familiarise yourself with the Mic instrument. Follow all instructions to ensure proper operation of the Mic instrument. Do not use any consumables, accessories, or external equipment other than that specified. Safety warnings must be adhered to at all times to avoid risk in personal injury and/or damage to the instrument. If the equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired. The advice given in this manual is intended to supplement, not supersede, the normal safety requirements established in the user's country.

Warning Symbols

The following safety warnings appear through this manual.

WARNING 	Electrical hazard.
WARNING 	Follow the instructions to avoid risk in personal injury.
CAUTION 	Follow the instructions to avoid damage to the instrument.
HOT SURFACE 	The temperature of the rotor may be above 40°C (104°F). To avoid injury, do not touch the rotor during a run or 5 minutes following an aborted run.
BIOLOGICAL HAZARD 	There is potential for exposure to infections agents when working with equipment used in molecular biology. To avoid exposure to such hazards ensure that proper personal protective equipment are worn and that laboratory best practise is adhered to.
ATTENTION 	Follow the instructions to ensure optimal instrument performance.

Proper Use Warnings

WARNING

Damaged Lid
Do not use the Mic instrument if the lid is broken or if the lid lock is damaged. There is a high risk of personal injury to the user through parts that are moving, electrically live, or are hot.

WARNING

Lethal voltages inside the instrument.
When the instrument is connected to line power, terminals may be live. Opening covers or removing parts is likely to expose live parts.

WARNING

Do not clean the chamber with flammable liquids.
The chamber can reach temperatures above 100°C (212°F). Any flammable liquids in the chamber could be a fire risk.

HOT SURFACE

Hot Surface
In the event of a user aborted run do not open the lid until the instrument has cooled. The rotor within the chamber could be above 40°C (104°F). To avoid personal injury, do not touch the rotor for at least 5 min.

CAUTION

Magnetic Locking Ring
Ensure that the magnetic locking ring is in place before starting a run to ensure the caps and tubes do not come out of the wells during the run.

CAUTION

Positioning the Instrument.
Do not position the instrument so that it is difficult to operate the disconnecting device.

CAUTION

Avoid spilling liquid into the chamber.
Any solution that spills onto electronic boards could cause a short circuit, damaging the instrument.

CAUTION

Do not obstruct the side vents.
Keep the side vents free from obstruction to prevent interference with the cooling of the instrument.

CAUTION

Do not move the Mic instrument during operation.
Movement may impair the proper function of the instrument resulting in poor data.

CAUTION

Power Connection
Avoid removing the power connector from the Mic instrument before the power indicator light is off on the power adaptor. Failure to do so may result in electrical arcing.

Type Plate Symbols

Regulatory Compliance Mark
This device is compliant with applicable ACMA technical standards for EMC.

FCC Declaration of Conformity
This device complies with Part 15 of the FCC Rules. Operation is subject to the following two conditions: (1) this device may not cause harmful interference, and (2) this device must accept any interference received, including interference that may cause undesired operation.

CE Marking
The device is in compliance with the essential requirements and other relevant previsions of Low Voltage Directive 2006/95/EC.

WEEE
Waste Electrical and Electronic Equipment Directive 2012/19/EU. Do not dispose of the instrument with general waste.

Biological Safety

Handle biological material with care and in accordance with the required safety regulations. Always wear safety glasses, gloves, and a lab coat. The user must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are suitably trained and not exposed to hazardous levels of infections agents[1].

Decontamination
Cleaning and decontamination of the instrument is necessary as a safeguard when the instrument and any accessories are to be transferred to the manufacturer or certified maintenance body for repair, service or returns.

Decontamination of Instrument

Surfaces of the Mic instrument, including the chamber and tube clamp, can be decontaminated using a solution of sodium hypochlorite (NaOCl). A solution containing 1 gL^{-1} available chlorine will be suitable for sanitation in a general lab environment; stronger solutions (5 gL^{-1}) are recommended when dealing with high risk situations[2].

Disposal of Waste

The disposal of wastes must be in accordance with all national, state and local health and safety regulations and laws.

Return Merchandise Authorisation

To ensure employee safety, Bio Molecular Systems requires that a Return Merchandise Authorisation declaration be completed and shipped with all returned items. Failure to comply will result in equipment being returned at the sender's expense.

Contact BMS via the email address support@biomolecularsystems.com to receive an RMA form. Complete the RMA form and attach a signed copy to the outside of the shipping container before shipping the instrument. Ship Mic back to:

Unit 5-3 Northward Street
Upper Coomera QLD 4209
AUSTRALIA

Please direct questions and enquiries regarding the RMA form to info@biomolecularsystems.com.

[1] Biosafety in Microbiological and Biomedical Laboratories, HHS (www.cdc.gov/od/ohs/biosfty/biosfty.htm).

[2] World Health Organization. Laboratory Biosafety Manual – 3rd ed. Geneva: World Health Organization; 2004.

Introduction

The Magnetic Induction Cycler (Mic) is a compact rotary based 48-well qPCR instrument that applies magnetic induction to achieve heating and forced airflow for cooling. Utilising our patented technology, Mic is fast enough to complete 35 cycles in 25 min[3]. The instruments come with either two or four detection channels with excitation and emission spectra that encompass the most common dyes used in qPCR.

With Bluetooth® connectivity to a PC, multiple instruments can be run via a single computer increasing sample throughput. The user friendly software is packed full of intelligent analysis features, including Relative Quantification and High Resolution Melting (optional). With an intuitive software layout, run setup and analysis become simple; and there are many quality control features to provide confidence in the final result. Most aspects of the software were designed to meet the MIQE[4] guidelines. And proper thought has been placed into applying the most up to date methods for qPCR analysis. We believe Mic will enable you to achieve fast, accurate and superior qPCR all within a compact format.

[3] Ten microliter volume reactions amplifying a robust assay using an antibody inhibited polymerase, requiring only a 30 s activation time. Longer denaturation times may be required for genomic DNA.
[4] *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments (Bustin *et al*. 2009).

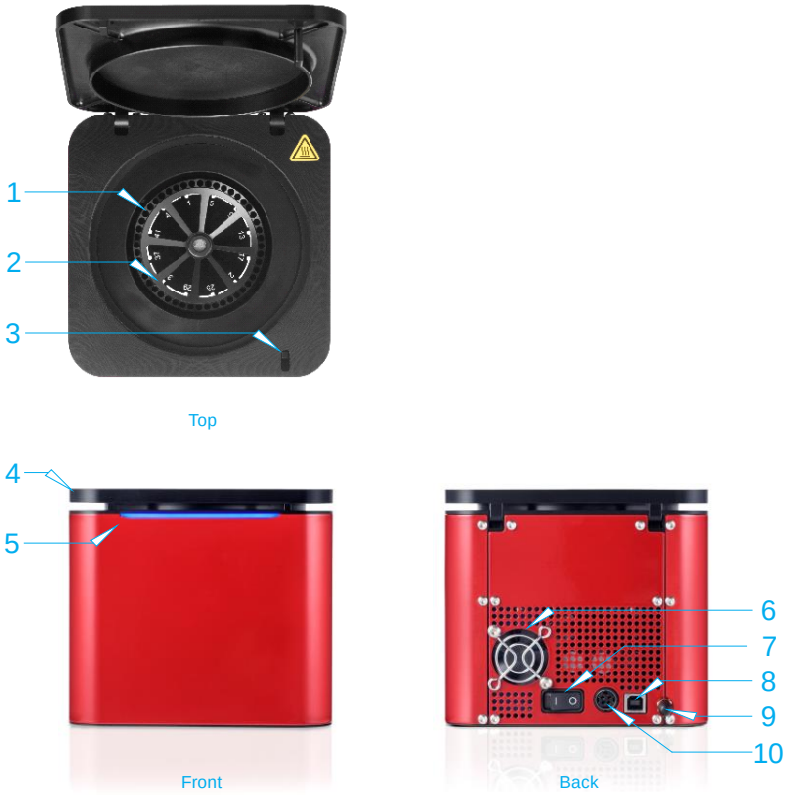
Instrument

Specifications

Physical	Dimensions	W: 150 mm, L: 150 mm, H: 130 mm (265 mm lid open)
	Weight	2.1 kg
Electrical	AC Input	100-240 VAC, 50/60 Hz 4.0 A
Thermal Performance	Temperature Accuracy	± 0.25°C
	Temperature Uniformity	± 0.05°C
	Ramp Rates	Heating: 4°C/s (fast mode) Cooling: 3°C/s (fast mode)
	Temperature Input Range	40 – 99°C
Optical	Detectors	High sensitivity photodiode per channel
	Excitation Sources	High energy light emitting diode per channel
	Channels	Green Ex. 465 nm Em. 510 nm filters Yellow Ex. 540 nm Em. 570 nm filters Orange Ex. 585 nm Em. 618 nm filters Red Ex. 635 nm Em. 675 nm filters
	Acquisition time	1 s
Reaction Vessels	Samples per Instrument	48
	Reaction Volume Range	10 – 25 µL
Operating Environment	Temperature	18 – 30°C
	Relative Humidity	20 – 80%

General Features

1	Rotor	Aluminium rotor with 48 sample positions and a tube location label to ensure correct loading of tubes.
2	Tube clamp	Safeguards against the tubes and caps from coming out of the rotor during a run.
3	Lid Lock	Mechanism that locks the lid shut during a run, to protect the user from moving parts.
4	Lid	Provides access to the rotor.
5	Power indicator	When illuminated blue, indicates that the instrument is powered ‘On’.
6	Extraction fan	Ensures cooling of electronics.
7	Power switch	Powers the instrument on/off.
8	USB cable inlet	USB connection to a PC.
9	Bluetooth® antenna	Wireless connection to a PC.
10	Power inlet	Connects to the power adaptor.



Consumables and Accessories

	Power adaptor REF 05562	External power supply for the instrument.
	Tubes and caps REF 60653	Strip of four reaction vessels with a volume range of 10 – 25 µL. Preloaded with silicone oil.
	Loading block REF 80418	Allows for the convenient loading of tubes with reagents and sample.
	Capping tool REF 90690	Allows for easy insertion of the caps into the tubes.

Installation

Unpacking the Mic Instrument

The following items are packaged within the Mic shipping container:

- Mic instrument (with tube clamp inside)
- Power adaptor
- Power cable
- 2 m USB cable
- Bluetooth® antenna
- Mic loading block and capping tool
- Mic tubes and caps (1000 reactions)
- USB flash dive containing copy of Mic software and manual
- Mic Quick Start Guide

Hardware Installation

Place the Mic instrument on a level surface.

Screw in the Bluetooth® antenna at the back of the instrument; or connect the instrument to a PC using the provided 2 m USB cable.

CAUTION

Instrument is not to be used with a USB cable greater than 3 m.

Plug the power cord into the adaptor and insert the adaptor into the back of the instrument.

CAUTION

To avoid electrical arcing, ensure that the power adapter is not plugged into a wall socket before plugging into the instrument.

Plug the power cord into a wall socket and switch the power on at the socket.

Power the instrument 'On' using the power switch at the back of the instrument.
An illuminated blue light at the front of the instrument will show the instrument is powered on.

Software Installation

Install the *micPCR*© software, located on the provided USB Flash drive, onto a PC.
Ensure that the PC meets the following minimum requirements:

- Windows® 7, 32 bit (English version) Operating System
- Intel i5 processor, 2.4 GHz
- 4 GB of RAM
- 1 GB free hard drive capacity
- Pointer device
- USB Drive
- Adobe® Reader® must be installed to be able to view reports in PDF format.
- Ensure that the PC has Bluetooth® enabled if this is the preferred connection

In the USB Flash drive menu, double click *micPCR.msi* software installer.

Follow the instructions that appear in the Setup Wizard.
If the computer is connected to a network, network policy settings may prevent you from completing this procedure. For more information, contact your system administrator.

When the software has been successfully installed, the *micPCR* software icon will appear on the PC desk top.

Open the *micPCR* software from the desk top icon.
The software will recognise the instrument via Bluetooth® or USB by displaying the *Instrument icon* in the tool bar (top right).
Multiple instruments can be recognised by the software and will be displayed in alpha numeric order.

Instruments connected to a PC via Bluetooth® and USB.

All connected instruments are now ready to be used.

Updating Software

Software and firmware updates are available for download at www.biomolecularsystems.com.
Please check the website periodically to see if new software and firmware updates are available.

Browse to the software *Downloads* page of the Bio Molecular Systems website.
To enter the restricted site, you will need to enter your user name and password, which will be provided to you upon registering your instrument online.

Download the *micPCR* software or firmware update setup file.

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

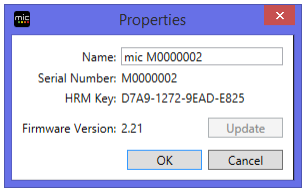
Upgrading to HRM

A HRM activation key, linked to an instrument serial number, will be provided upon purchase of the HRM upgrade module.

Start the *micPCR* software and ensure that the instrument is in communication with the PC.

Select the instrument requiring HRM activation then select *Properties* (see XXX).

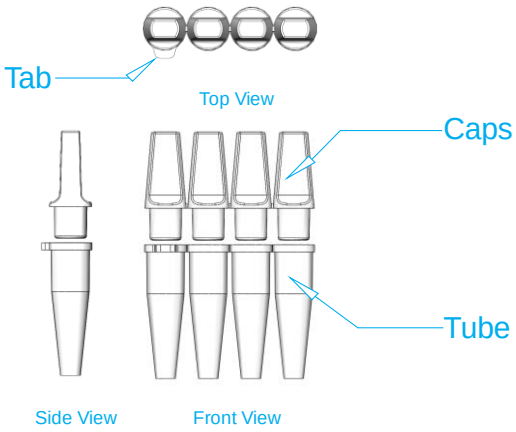
Enter the activation key in the provided field and select OK.
The instrument will now have HRM enabled. Only data collected on this instrument can be analysed using HRM.



Getting Started

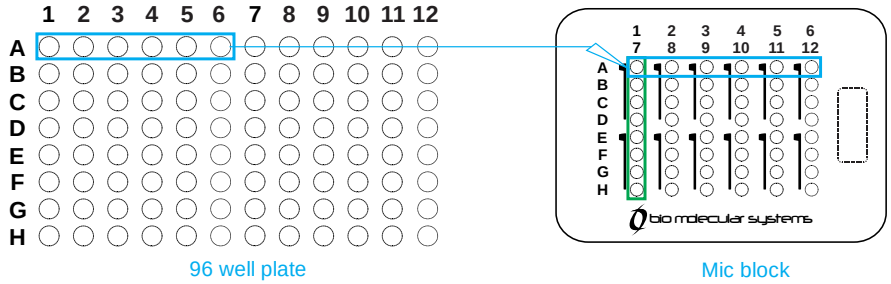
Loading Tubes

Each tube is part of a strip of four, with the first tube having a small tab to ensure the strip is loaded into the instrument correctly. Each tube is preloaded with silicone oil, which acts as a barrier to prevent evaporation and condensation, thereby improving reaction performance and removing the need for a heated lid. The allowable range of total reaction volume is 10 - 25 µL.



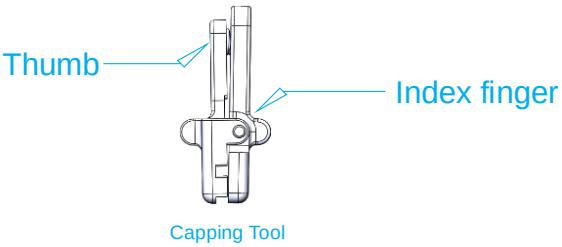
The caps are designed to fit tightly into the tubes. Each tube is preloaded with silicone oil that acts as an overlay during the reaction, limiting condensation. A tab on tube one is used as a marker to ensure the tubes are correctly oriented into the loading block and instrument.

Use the provided loading block to pipette reagents and samples into each tube.
Place the tubes into the loading block, ensuring the tube tab matches the white tab on the loading block.
The loading block is also compatible with a multichannel pipette (8-channel). For this strategy the loading orientation switches to across the top of the loading block (A1, A2, A3…) instead of the standard down orientation (A1, B1, C1…). The software has provision to change the display of samples based on the type of layout selected.
Note: Ensure that only the first six tip positions are utilised and not the full eight.



Wells highlighted in green follow the standard loading orientation (A1, B1, C1...) and wells highlighted in blue follow the multi-channel pipette loading orientation (A1, A2, A3...). The loading strategy will allow you to load half a 96 well plate per loading block.

Once the tubes are loaded, properly fit the caps to ensure the tubes are sealed.
Use the provided capping tool to help with fitting caps properly and avoid cross contamination.



Capping Tool

The tool is designed to clamp down on a strip of four caps. With your thumb and index finger on either side of the tool press the caps down into the strip of four tubes until firmly in place. Remove the tool by unclamping the caps.

The caps can be removed later to access the post PCR reaction for downstream applications such as gel electrophoresis or DNA sequencing.

ATTENTION

Ensure that post PCR amplicons are handled away from a pre-PCR environment to avoid contamination issues.

Place the reaction tubes into the rotor keeping the tube tab in line with the marker located on the rotor label.

Load *water tubes* in unused wells.
No tubes, empty tubes and tubes with different volumes of liquid all have different thermal loads on the metal rotor. Variations in thermal load around the rotor can cause significant thermal gradients both at static temperatures and during ramping, resulting in increased variability in results.
As the oil overlay prevents evaporation, these *Water Tubes* can be stored and reused for over a week.

ATTENTION

To achieve optimum temperature uniformity it is very important to load tubes, pre-filled with water, into the unused wells of the rotor using the same volume as that of the reaction tubes.

After loading all the tubes, place the tube clamp at the top of the rotor.
The tube clamp will safeguard against any of the tubes or caps coming out of the rotor during a run.

Once the lid is closed the instrument is ready to be run.
Failure to close the lid will prevent the run from starting. This is to prevent injury to the user and/or damage to the instrument.
When the run begins the lid will be locked into place to prevent it being opened.

Removing Tubes

Once the run has completed and the instrument has cooled down, the lid lock will disengage allowing you to open the lid.

Hot Surface
If the lid lock has disengaged due to a power failure or fault prior to the run stopping, please do not open the lid for at least 5 minutes until the chamber has cooled. The rotor within the chamber could be above 40ºC (104ºF). To avoid personal injury, do not touch the rotor for at least 10 min.

Remove the tube clamp and place it to the side.

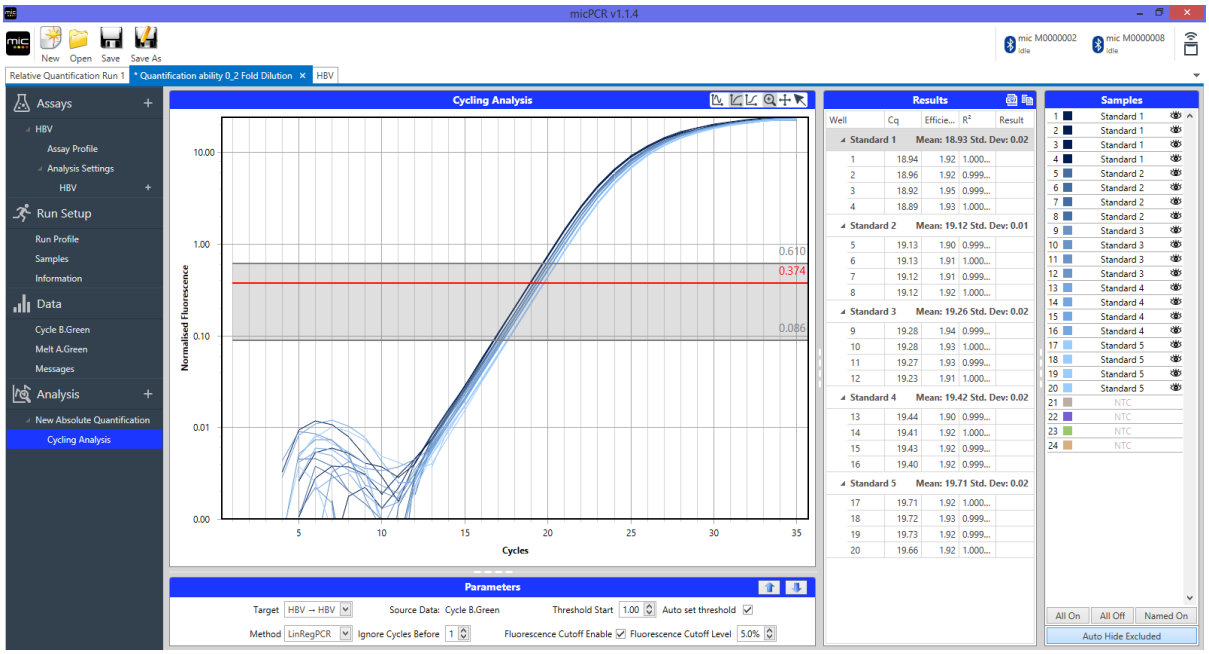
Pull the tubes out of the wells.

Ensure that you put the tube clamp back into the chamber before closing the lid to ensure it is not lost or damaged.

Software Overview

The software is divided into a number of sections:

1. Tool bar
2. File tabs
3. Navigator bar
4. File active windows
5. Samples selector



Tool Bar

The top section of the user interface is referred to as the *Tool bar* and consists of the following:

Mic: access to *micPCR Manual, Create Support Package* and *About micPCR*.

New: create a new *Assay* or *Run* from a drop down menu.

Open: open a saved *Assay* or *Run* from a file directory.

Save: save an open *Assay* or *Run*.

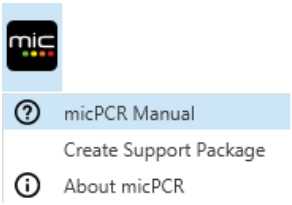
Save As: save an open *Assay* or *Run* under another file name.

Instrument: instruments in communication with the PC are displayed in the tool bar.

Instrument Communication: detect available instruments via USB or Bluetooth®.

Mic Icon

The Mic icon is used to access the following options:



micPCR Manual: an electronic version of the user manual is stored within the software.

Create Support Package: create a support package after experiencing any fault with the software or hardware. The support package contains a compressed log file of the run. Select a folder to save the support package to. Email the zipped support package file to support@biomolecularsystems.com.

About micPCR: information about the version of micPCR software.

Open Assay or Run Files

There are two file types:

Assay File : contains all the information regarding a set of targets for a run.

Run File : contains the assays used, run profile, sample annotation, raw data and analysed data.

Instrument Communication Icon

The instrument communication icon is used to search for nearby instruments via both Bluetooth® and USB.
On software start up the icon will flash automatically indicating the software is searching for instruments.
The maximum range for the Bluetooth® antenna is approximately 7 m with no obstruction from solid walls.

Instrument Icon

Instruments in communication with the PC will be displayed in the tool bar as an *Instrument icon*.
A Bluetooth® or USB symbol will indicate the type of communication achieved.
The serial number or name of the instrument is displayed next to the communication symbol.
The status of the instrument is also displayed beneath the name:

Idle: instrument can be used to start a run.

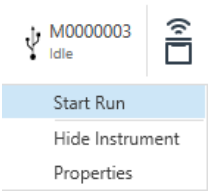
Setup: someone has transferred run information to the instrument from another connected PC but has not yet started the run. This instrument cannot be used until the run has completed.

Running: instrument is running and cannot be used until the run is completed.

Offline: instrument has lost communication with the PC.

Reconnecting: instrument is regaining communication with the instrument during a run.

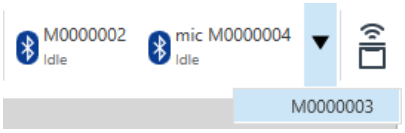
Click on the instrument icon to display the following options:



Start Run: runs begin by selecting the *Start Run* option. The *Start Run* option will only appear when a *New Run* has been initiated.

Hide Instruments: select *Hide Instrument* if you do not wish to display a particular instrument in the software. Use this option if you want to avoid cluttering your PC with other instruments you are not using but are in communication with.

Unhide: to unhide an instrument, select the *down triangle* to display a list of hidden instruments; then select the instrument you wish to unhide.



Properties change the name of the instrument, or upgrade to HRM; and there is also information regarding the serial number and firmware version.

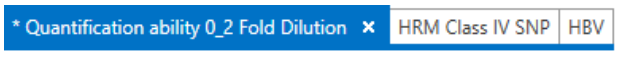
Update Firmware: updating of the instrument firmware. The option is only available after specific micPCR software upgrades.

Reconnect Run: lets the PC reconnect to a run following a dropout when using Bluetooth®.

Recover Run: obtains the run from the instrument if connection has dropped out and the run has completed. The instrument will store the data until such time.

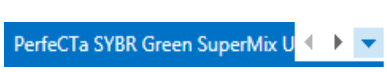
Reconstruct Run: allows the user to view the run on another PC connected to the instrument that did not start the run.

File Tabs

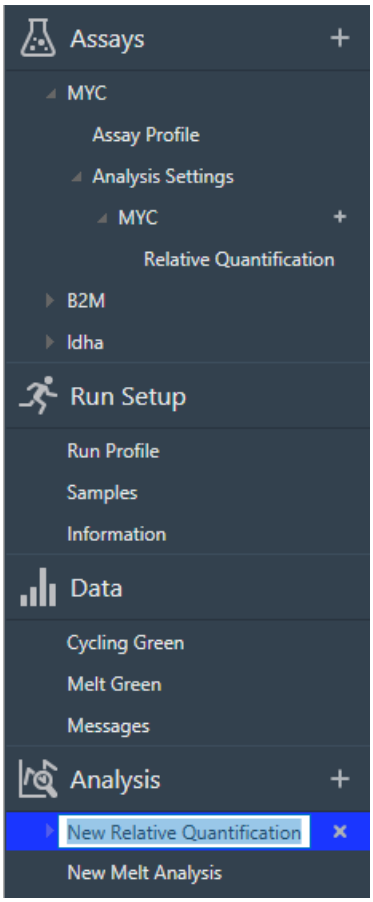


Every open file will be displayed with its name on a tab. Multiple files can be open at the one time. The file being displayed in the main window will be highlighted in blue. Files that need to be saved will have an asterisk just before the file name. If a file is linked to a run in progress, selecting the instrument running it will open the associated tab.

Use the down arrow to view files that might be out of view if multiple tabs are open at the one time.



Navigator Bar



To the left hand side of the main user interface is the *Navigator bar*. The Navigator bar allows you to view the different sections for an *Assay* or *Run*.

Some sections contain subsections that can be viewed by expanding the navigator tree.

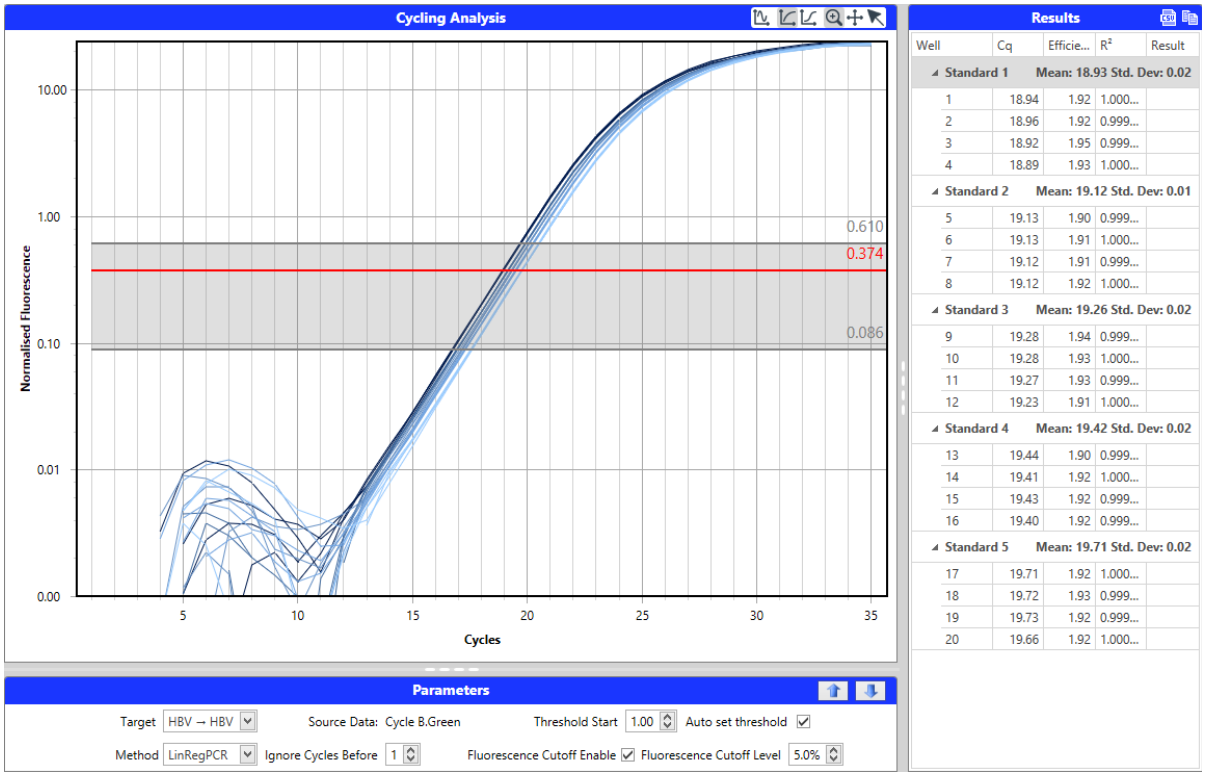
Sections that are open in the main window will be highlighted in blue.

Analyses can have their names changed within the Navigator bar.

Remove Assays and Analyses by using the delete icon.

File Active Windows

In the central area of the user interface are segmented windows that are active for a specific section of the navigator bar.



Raw *Data*: the data is updated in real-time during the run and is available after the run has completed. The graphs are scaled automatically during a run and can be scaled manually by expanding or contracting each axis.

Analysis graph: analysed data displayed in a specific graph type depending on the analysis chosen. The graphs can be manually scaled by moving each axis.

Analysis *Parameters*: can be changed to optimise for the target being analysed (see XXX).

***Results* table**: numerical representation of the analysed data. The table can be setup to display the samples as replicates, with mean and standard deviation, or as individual wells (see XXX).

CSV Export or Copy to Clip Board

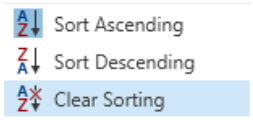
Each *Result table* will have two options for copying the data for easy export to third party software:

CSV: save the results table as a CSV file.

Copy to Clip board: copy the results table to the clipboard, then paste into another third party software such as Microsoft® Word®.

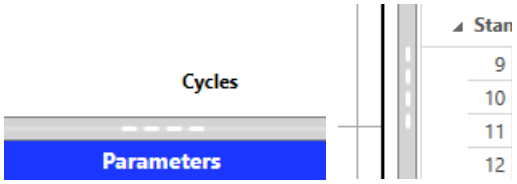
Result Table Organisation

Each column can be sorted in both Ascending and Descending order by clicking on the column or by right clicking on the column.
To undo sorting, right click on the column and select Clear Sorting.



Sizing Bars

The width or height of these windows, relative to each other, can be adjusted using the sizing bars.



Graph Display Functions

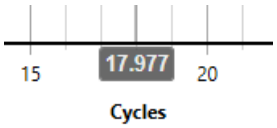
There are four graph display functions available: 

Zoom: magnify a chosen area to view more detail within the graph. To zoom back out, double click anywhere on the graph.

Pan: combining the *Zoom* function with *Pan* allows you to move the display around a magnified field of view allowing you to locate and focus on specific areas of the graph.

Select samples: only the selected samples will be displayed on the graph.

Cross hairs: allows you to find the coordinates for the x-axis and y-axis for any displayed graph. Values are displayed in a grey text box along each axis.



Parameter Settings

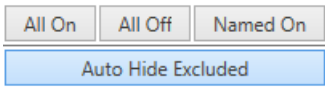
Parameter settings differ for each analysis type. They allow you to pick the appropriate algorithms or adjust values used to calculate the final result from the available data set (see XXX). Parameter settings can be stored or reset to their original values at any time using the two following options:

Save settings to Assay: uploaded any changes made to the parameters in the run analysis to the assay file. Use this option to apply the updated parameters for future analysis of the same file. Save the *Assay* file to use the parameters for new runs setup in the future.

Load settings from Assay: re-set the parameters to the original assay file values.

Samples Selector

Deselect and select specific samples using the *Samples selector* located to the right hand side of the user interface. Select/deselect individual samples by clicking on the sample bar. Alternatively, select just the named samples using the *Named On* button, or select all 48 samples by using the *All On* button, or deselect all of the samples by using the *All Off* button.



Hovering the mouse curser over a sample in a graph will highlight the sample by drawing the line thicker and displaying the name of the sample in a small text box along with the well number. The sample bar in the *Samples selector* is also highlighted in black.

Use the sample selector to remove or reinstate samples from analysis. The removal or addition of samples from analysis may change the position of the W-o-L and therefore the automatic cycle threshold. Poorly amplified samples can affect the performance of the LinRegPCR algorithm, preventing it from determining a W-o-L. Removal of such samples from the analysis may allow for the determination of the W-o-L.

To only hide a sample from view within a graph, and not remove it from the analysis, use the *Visual* icon located on the sample selector.

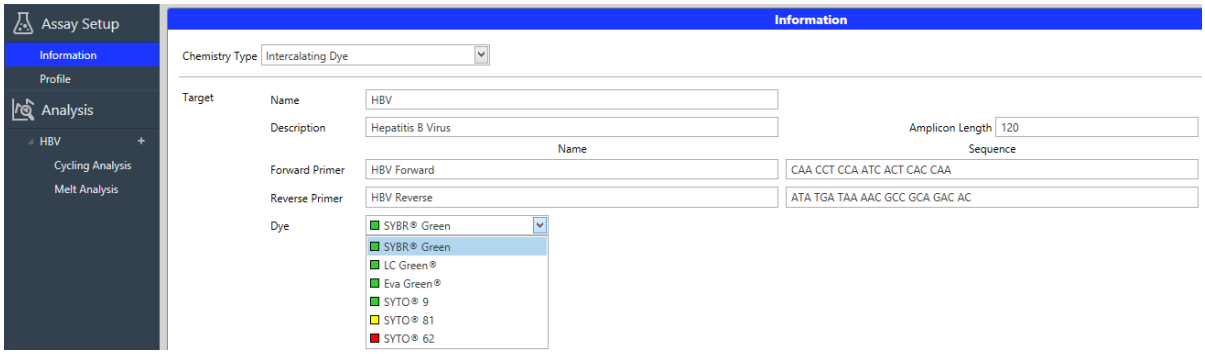
Creating a New Assay

The *Assay* contains information regarding the target amplicons and the qPCR conditions and the analysis type required for the assay (e.g. *Relative Quantification*) along with various analysis parameters. Once an assay is setup it is stored in a library from where it can be located and added to any future run. A new run can begin by simply selecting the assay(s) required, which contains all the necessary information to achieve both the run and analysis.

Select *New* from the tool bar menu and then *Assay* from the drop down list.

Assay Setup

Information (Optional)



Select the *Chemistry Type*.

Choices include *Intercalating dye*, *Dual hybridisation Probes*, *Hydrolysis Probes*, *Molecular Beacon* probes, and *LUX® Primers*~~, Plexor®, or Scorpion® probes~~.

The type of chemistry selected will also set the default *Assay Profile*.

Enter the name of the amplicon target.

If using multiple targets for the one assay (multiplexing), select the *Add* button to setup another *Target*.

Multiple targets for the one assay are not permissible when using intercalating dyes.

Enter the *Target Details*.

Provide as much detail as required to describe the target. This is especially important when working with splice variants. Try to include details such as intron and exon boundaries, and/or species.

Enter the length of the amplicon.

Enter the *Name* and *Sequence* information for each of the oligonucleotide primers.

Provide notes regarding the location of the primers, for example exon location, in the name.

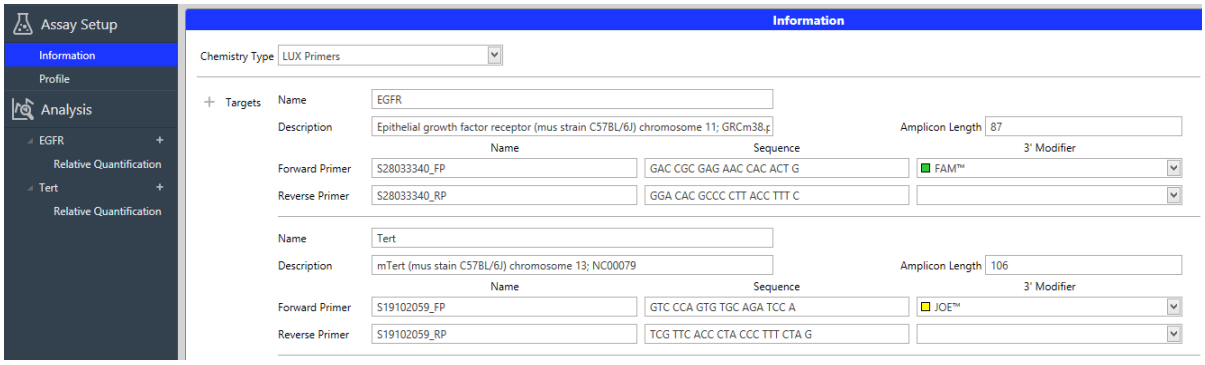
Enter the sequences in a 5-prime to 3-prime direction.

For *Intercalating Dye* chemistry select the reporter dye used.

This will set the channel required for acquisition.

If using *LUX Primers* select the reporter dye used in either the forward or reverse primer sequence.

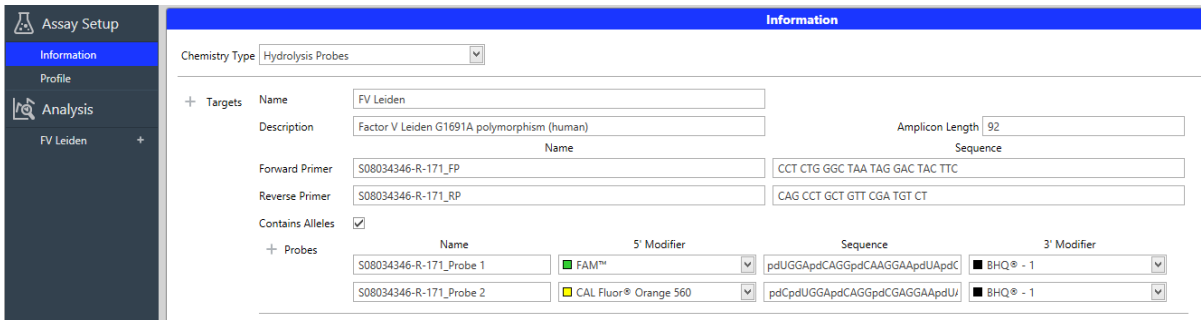
This will set the channel required for acquisition.



When using *Hydrolysis Probes* or *Molecular Beacons* enter the name and sequence information for the probe.

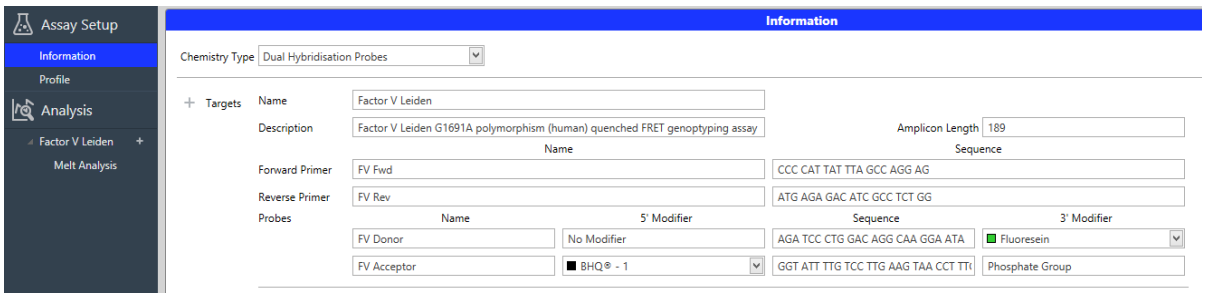
You can add multiple probes for the same target if required for *Allelic Discrimination*, by ticking the *Contains Alleles* check box. Select the Add button to setup another probe. A maximum of four probes can be selected.

Select the reporter dyes and quenchers used. This will set the channels required for acquisition. Probes with the same acquisition channel will not be allowed.



Enter the name and sequence information for each probe when using Dual Hybridisation Probes.

Select the reporter/donor dye for the 3′ position in the first probe, which will set the channel required for acquisition, and the 5′ quencher/acceptor in the second probe. The second probe will also be labelled with a phosphate group at the 3′ end, which prevents the probe from initiating polymerase extension.

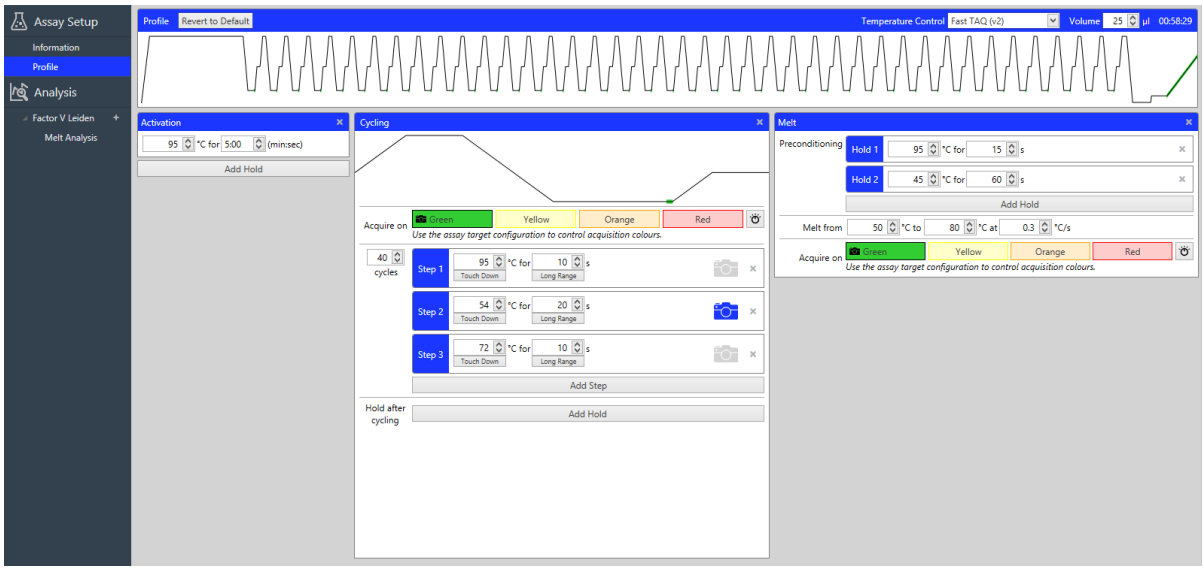


Assay Profile

A generic assay *Profile* is provided at the beginning. Modify the profile to suite the assay.

The generic assay profile displayed will depend on the type of chemistry and reporter dye selected in the *Information* section.

To return back to the original generic profile, use the *Revert to Default* button.



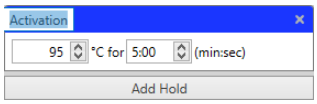
Initial Holds

Enter as many *Hold* steps as required.

Hold steps may be required for reverse transcription, UDG activation, activation of the hot start polymerase, and denaturation of the DNA template.

Enter the hold temperature in degrees centigrade and the hold time in minutes and seconds.

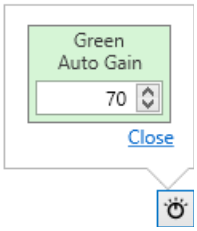
You can name each of the holds in the title bar by double clicking on the name.



Note: Genomic DNA may require longer hold times to ensure complete dissociation of the complementary strands and unravelling of tertiary structures. Failure to fully denature the genomic DNA may result in a significant drop in baseline fluorescence at the start of the run, late amplification and poor reaction efficiencies.

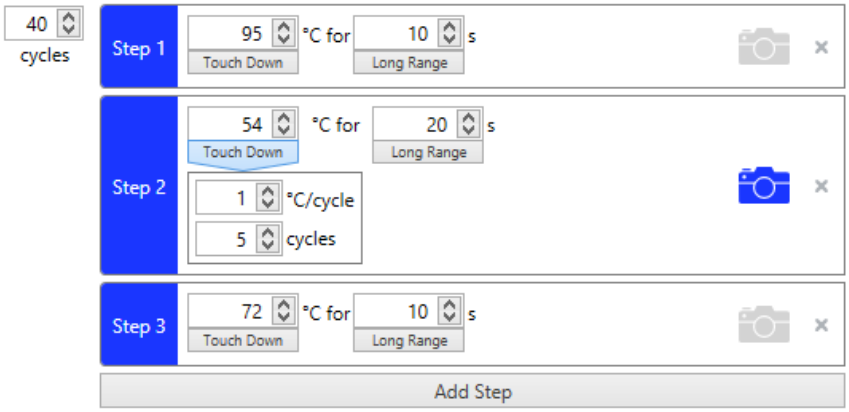
Cycling

Adjust the gain settings for each channel by selecting the *Adjust gain settings* icon.
A pop up window with all the channels selected to acquire on will appear to allow you to select the appropriate background position.



When using chemistries that start with a high background and reduce in signal during cycling, such as quenched FRET dual hybridisation probes or Plexor®, set the baseline high. The default option for such chemistries is 70 units. The instrument will adjust the gain of the selected optical channel such that the baseline is around 70 units or until the maximum gain and scale are reached.
For all other chemistries that begin with a low baseline and increase in signal during cycling, select the low baseline. The default option for intercalating dye chemistry is 3 units, and for all other chemistries it is 10 units. The instrument will adjust the gain of the selected optical channel such that the baseline is around 3 or 10 units or until minimum or maximum gain is reached (default option).

Enter the number of *Cycles* required.



Program the cycling parameters by either adding or removing *Steps*.
Use the *Add Step* button to provide an additional step or the *Delete* button to remove a current step.
Enter a temperature and a hold time for each step.
Minimum time allowed is 1 s and a maximum of 120 s.
The allowable temperature range is from 40 to 99°C.

There are two additional options available during cycling, *Touchdown* and *Long Range*.

Touchdown: can be enabled to decrease the temperature during the initial cycles.
Set the number of cycles and the decrement in temperature per cycle.
Touchdown will increase the specificity of primer binding at the beginning of cycling by applying a more stringent temperature for annealing. And then increase amplicon yield later by reducing the stringency of primer annealing.

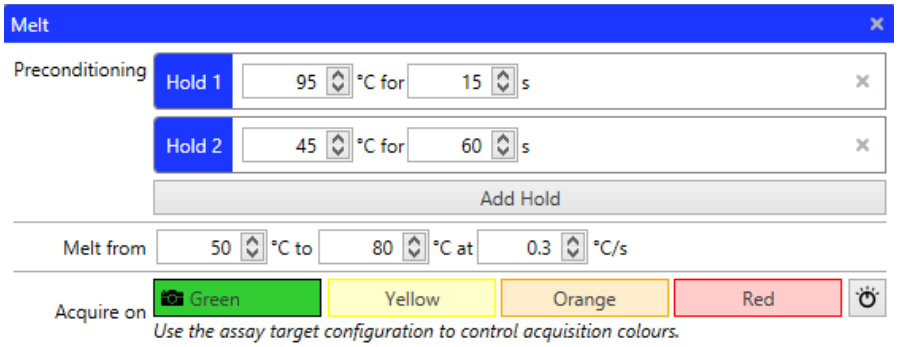
Long Range: can be enabled to increment the hold time of the selected step**.**
Set the number of cycles and the increment in time per cycle.
The concept of long range is similar to touchdown, utilising the annealing hold time rather than temperature.

Select the step to acquire on by selecting the *camera icon*.
Acquisition should primarily occur following the annealing or extension steps. However, only one step can be chosen for acquisition.

Add a hold after cycling if required.
A hold after cycling could be applied to ensure complete extension of amplicon prior to post PCR analyses such as gel electrophoresis or sequencing.

Melting

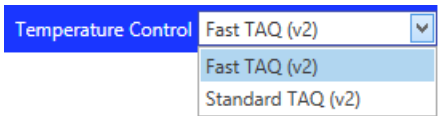
Modify or remove a *Melt* from the run.
Melts will only be added for specific chemistries compatible with melt analysis.
Only one channel will be acquired per melt. A second melt is applied for a second channel; up to four per run.
Add preconditioning hold steps to improve the melt performance (e.g. 95°C hold for 20 s prior to the melt).
Enter the start and end temperatures.
Enter the ramp rate required. The minimum allowed ramp rate is 0.1°C/s. Ramp rates greater then 1°C/s will generate data that may not be substantial enough to provide meaningful information.



Adjust the gain settings for the melt acquisition.
Only channels selected in the Information section will be available in the melt.
Adjust the gain setting by selecting an appropriate baseline to start the melt with. For typical melts that start with a high background the default baseline is set to 70 units. For chemistries that have an increase in signal during the melt, the default baseline is set to 30 units.

Temperature Control

Determine if the run will be using a *fast Taq polymerase* or a *standard Taq polymerase*.
If using a standard *Taq* polymerase (not specified by the manufacturer to be compatible for fast cycling), the instrument can slow the rate of heating to allow for sufficient time to complete extension at the optimum temperature range for most *Taq* polymerases (70 – 80°C). The advantage of using the *Standard Taq* option is that you can avoid the need to use longer annealing steps, which can reduce analytical specificity; and avoid the need to run a third step at 72°C, thereby saving time. As the Mic instrument is designed for rapid cycling, the *Fast Taq* temperature control is the default option.



Select the *Standard Taq* option of the *Temperature Control* if using a standard *Taq* polymerase with two step cycling and short annealing times (< 20 s).
Due to the fast temperature ramping speeds possible on the Mic instrument, failure to apply a slow rate of heating for standard *Taq* polymerases, without using longer hold times at anneal (> 20 s) or three step cycling, may result in poor amplification, with reduced analytical sensitivity and amplification efficiencies.

Reaction Volume

Select the *Reaction Volume* to be used.
To ensure optimal cycling performance, it is important to select the correct volume. The volume selected will determine the appropriate thermal model the instrument should use for heating and cooling.
Do not use different reaction volumes in the same run. Thermal uniformity will be optimal when all samples contain the same volume. See XXX regarding the use of *water tubes* as another measure to ensure the highest level of temperature uniformity.

The time to complete the run is also displayed next to the reaction volume and is updated if there is any modification made to the profile.

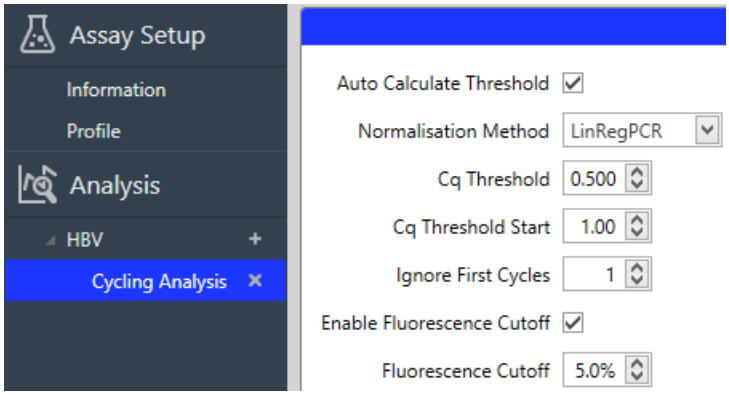
Assay Analysis

This feature enables you to store all analysis parameters within the assay, thereby avoiding continually having to edit parameters for each new analysis.

Select the *Analysis* required for the assay by using the *Add* button (Optional).
The choices of analysis are: *Cycling Analysis*, *Melt Analysis*, *Relative Quantification*, and *HRM*. There are no parameter settings available for Absolute Quantification or Standard Curve in the Assay Setup.

Cycling Analysis

The determination of C_q values and individual sample efficiency is achieved using *Cycling Analysis*. There are a number of parameters that can be set for *Cycling Analysis,* all of which will influence the calculation of C_q values and efficiency. Cycling analysis is used for other analysis options including Relative Quantification, Standard Curve, and Absolute Quantification. For these primary analysis applications, it is recommended to additionally set the appropriate parameters for Cycling Analysis using data from optimised reactions. The following parameter settings can be stored in the assay for Cycling Analysis:



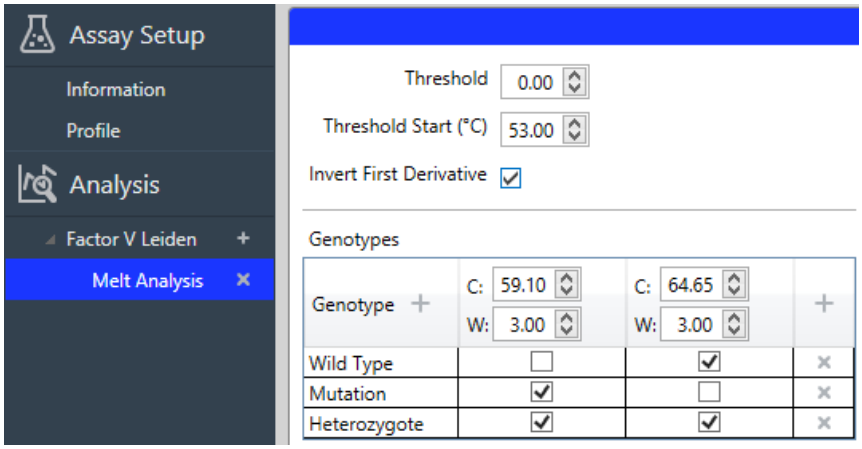
Determine if you want to set the cycle threshold yourself or change the cycle *Threshold Start*.
Uncheck the *Auto set threshold* box to manually set the cycle *Threshold level*.
For some data it may also be important to start the cycle threshold after a region of baseline interference.

Select an appropriate baseline correction method.
There are four options to select from the *Methods* drop down list.
If using *Dynamic* or *Fixed* length baseline correction, it is possible to *Ignore First Cycles* to avoid significant changes in baseline that may influence the result.

Set the Fluorescence Cut-off level.
Any sample with a fluorescence signal change below a user defined percentage of the sample with the maximum signal change will be excluded from analysis.

Melt

Melt analysis can be used as a quality control for cycling analysis by checking for non-specific products or amplicon contamination. It can also be used to genotype samples based on the differences in melt temperature between alleles. Chemistries compatible for melt analysis include intercalating dyes, dual hybridisation probes, Plexor®, and molecular beacons.



Set the temperature *Threshold* and *Threshold Start* for the melt.

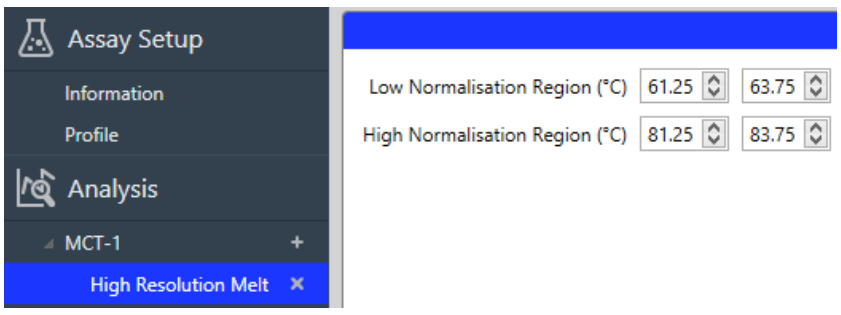
Invert the data if using chemistries such as quenched FRET dual hybridisation probes or Plexor®.

Set the T_m *bins* for each expected allele if using Melt analysis for genotyping.
The bins are the expected temperature peak values for each allele.
Also enter the expected range for each bin. The range should take into account the melt reproducibility and repeatability of the assay.
It is recommended that the bins be set following assay optimisation.

Organise Genotypes.
Add genotypes and link them to a *T_m Bin*.
Heterozygotes will use both bins.
You can add more genotypes by using the *Add* button.

HRM

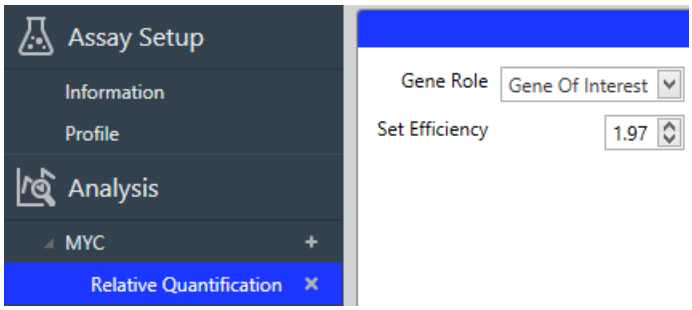
High resolution melting lets you determine differences in sample melting characteristics by using normalised melt curve data.



Enter the normalisation regions for the start and end of the melt curves.
These regions will be used to normalise the melt curves for HRM.

Relative Quantification

Relative quantification allows you to analyse differences in gene expression for a given group of samples relative to another control group; for example, measuring gene expression in response to a drug. To achieve relative quantification, an endogenous reference gene must also be run in parallel with the gene of interest. Furthermore, the efficiency of each gene must be taken into consideration when calculating expression values.



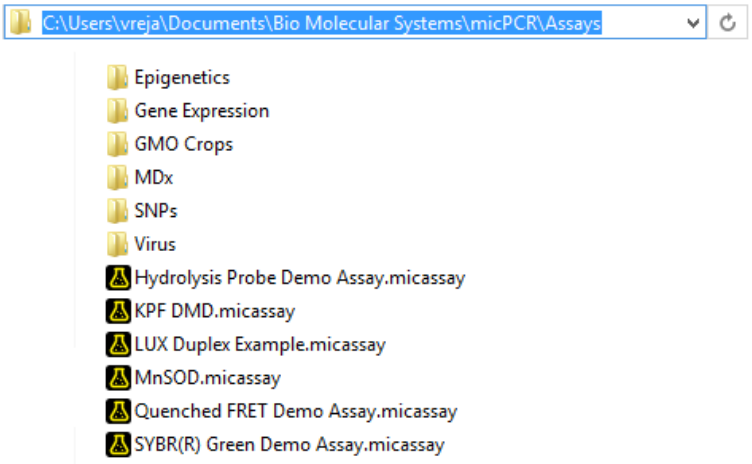
Select the *Gene role* for the target as either *Gene of Interest* or *Reference Gene*.

Enter an efficiency value for the target rather than using individually calculated efficiencies for each sample or using a standard curve in each run (Optional).
The *Set efficiency* value can be derived from previous experiments, or use the default value of 2 (not recommended).

Note: ensure that the correct Cycling analysis parameters are set for each target.

Saving a New Assay

Once all the parameters have been entered for the assay, the assay should be saved.
Assays will be saved into the following directory of your PC: Libraries/Documents/Bio Molecular Systems/micPCR/Assay.
You can create and store assays in subfolders of the main assay library.
It is important to have all the assays stored together so that finding them and linking them to a sample in a run will be easy.

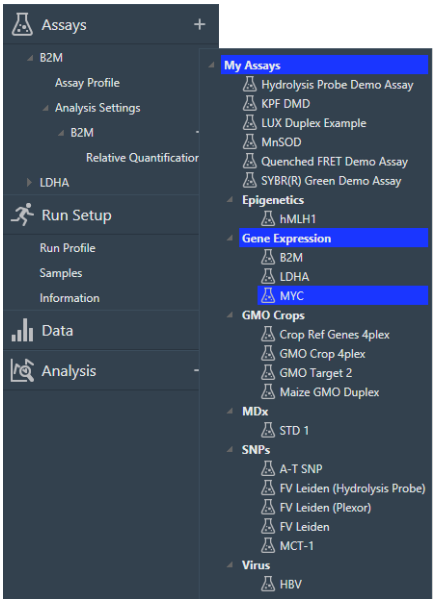


Creating a New Run

Select *New* from the tool bar menu and then *Run* from the drop down list.

You have the option to create a run using saved assays (recommend) or without any defined assays. Relative Quantification analysis will not work without pre-defined assays.

Adding Assays



Select the *Assays* required for the run by selecting the *Add* button.

Select from your library of assays displayed next to the navigator bar.

The *Information*, *Assay Profile* and *Analysis Settings* can be viewed and edited once the assay is selected.

Remove assays by using the delete button next to the assay name.

The *Run Profile* is based on the first assay that is selected. Any following assay selected, which fails to meet the *Run Profile* temperatures and hold times, temperature control, or reaction volume will be determined to be non-compatible.

Assay Profile Compatibility

If an assay is selected that has a *Profile* not compatible to the first assay, the software will bring up the following warning message:

"The selected assay is not compatible with the run. The assay's profile and current run's profile do not match and cannot be automatically adjusted. Please indicate how you wish to proceed:"

Select from the following options:

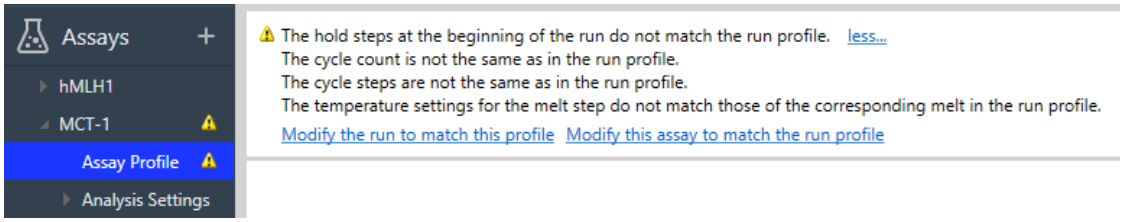
- Modify Run: The run's profile will be modified to make it compatible with the selected assay. This may cause existing assays to become incompatible.
- Modify Assay: The assay's profile will be adjusted to match the run. This may also add melt steps to the run's profile if required by the assay.
- Add Without Modification: The selected assay will be added without modification. The assay will remain incompatible until it or the run's profile is modified.
- Cancel: Do not add the assay to the run.

Non-compatible assays are reported with a caution symbol.

Specific reasons for the non-compatibility are reported below the symbol. You can also enter the Profile for the incompatible assay and select *more…* to view all of the reasons. In the area you have the choice to:

Modify the run to match this profile: The run's profile will be modified to make it compatible with the selected assay. This may cause existing assays to become incompatible.

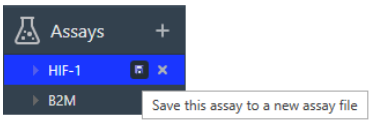
Modify this assay to match the run profile: The assay's profile will be adjusted to match the run. This may also add melt steps to the run's profile if required by the assay.



Note: Test any modified profiles on a small subset of samples to confirm these changes are valid, before running experiments with a large number of samples to avoid major loss of valuable samples and reagents. The consequences might be suboptimal performance of the qPCR leading to poor results.

Updating Changes to an Assay

Further changes can be made to the assay including the *Information* fields and *Analysis Settings*. These changes can be saved to a new or existing *Assay file*, by clicking on the *Save* icon located next to the name of the assay.



Run Setup

Run Profile

You can modify the *Run Profile* independently of the assays. Reasons for changing the run profile include the testing of a new polymerase with a different activation time, or adding an extra cycling step to improve template yield. Modifying the *Run Profile* will save time, compared with changing individual *Assay Profiles* when using multiple assays in the run. The software will pop up a warning that the selected assays are not compatible with the *Run Profile*.
Note: It is always important to validate the changes using a small sample number to avoid loss of valuable sample and reagents.

Alternatively, you may choose to start a run without using Assays.

Modify the *Run Profile* if required (Optional).
The values displayed in the Run Profile are the conditions that will be used during the run.

Select the channels to *Acquire on.*
Multiple channels can be selected.
If running a conventional PCR then no acquisition channel is required. A warning will be displayed if no channels are selected. Acknowledge the warning if you wish to proceed with no acquisition.
Channels will be preselected if the reporter dyes have been selected for individual targets in the

Information section of the *Assay Setup*.
Only one channel can be selected per melt. Another melt will be required to apply a second channel.

Adjust the gain settings for each channel by selecting the *Adjust gain settings* icon.
To perform gain adjustment, you have the option to use *All Tubes* (sample with maximum signal intensity is used) or select a specific sample from the drop down list. This option is only available in the Run profile editor.

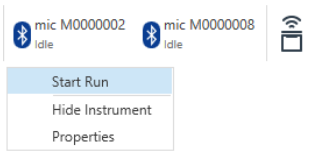
Determine if the run will be using a fast *Taq* polymerase or a slow *Taq* polymerase.
Select the *Slow Taq* option of the *Temperature Control* if using a non-fast Taq polymerase with two step cycling and short annealing times (< 20 s).

Select the *Reaction Volume* to be used.

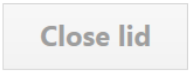
The time to complete the run is also displayed next to the reaction volume and is updated if there is any modification made to the profile.

Starting the Run

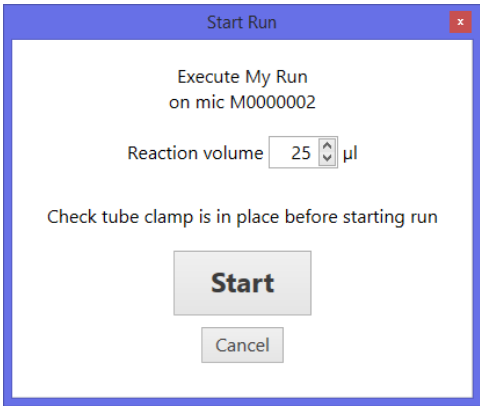
Select the *Instrument* you wish to use for the run in the tool bar.
Only Idle instruments can be selected to start a run.



Once the instrument is chosen, begin the run by selecting the *Start* option from the drop down list.
A confirmation dialogue box will appear.
Ensure that the tube clamp is in place to prevent the tubes and caps from coming off during a run.
Ensure that the lid is closed prior to starting the run. A lid sensor will detect if the lid is open and will prevent the instrument from starting, while a warning will notify you of the fact.



Double check that the volume displayed is correct; if not, select the appropriate volume.



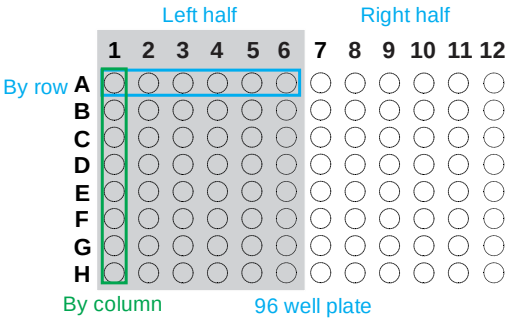
To execute the run click the *Start* button in the Start Run dialogue box.
The instrument will automatically lock the lid and spin the samples down. Then the run profile will begin.

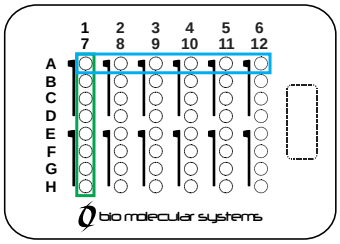
Samples Editor

The *Samples editor* is displayed in a table format and allows you to annotate your samples. Samples can be annotated before, during or after a run. Failure to properly annotate samples can affect analysis.

Well Layout

The well layout can accommodate loading from a 96 well plate using a multichannel pipette, where the orientation switches from columns to rows. To ensure the software displays this change, select from the options below.





Display wells as either numerical (1, 2, 3…) or alpha numeric (A1, B1, C1).

If loading from a 96 well plate select if the samples are displayed from the left half of the 96 well plate (A1 – H6) or the right half (A7 – H12).

If loading from a 96 well plate using a multi-channel pipette, change the orientation of the display from '*…by column'* to '*…by rows'*.

Filling Cells

Cells can be filled individually or in groups.
Using the *Enter* key on your keyboard will move to the next cell down.
Using the *Tab* key will move to the next column.
Use the delete key to clear a cell.
Copy and then paste names and concentrations from other software programs (e.g. Microsoft® Excel®).

Colours

Select the *Colour* you want for each sample (Optional).
Chose any colour from the colour pallet or generate your own colours using the colour chart.
To create a gradient select the first colour and highlight all the way down to the last colour required, and then click the *Auto fill* icon  .

Name

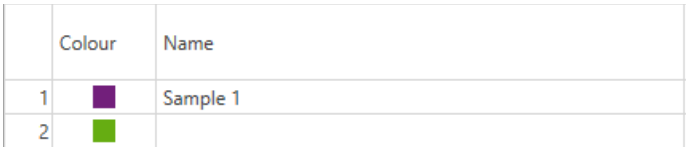
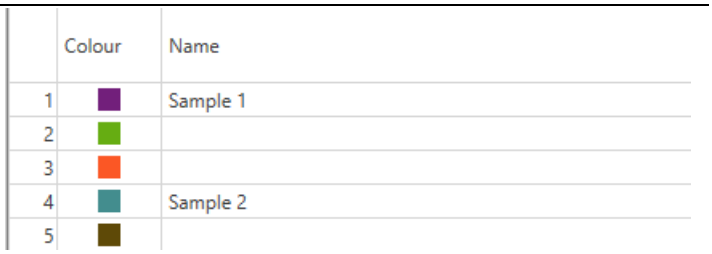
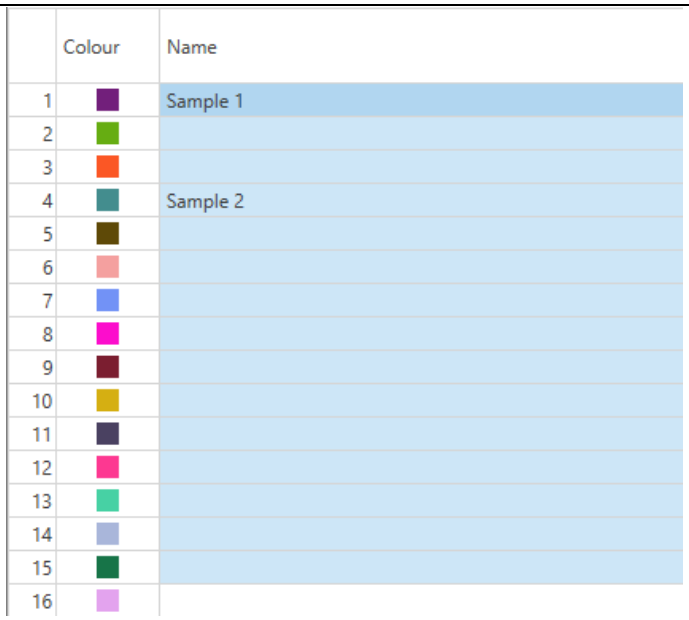
Enter the *Name* of each sample.

Samples with the same characters and assay will be treated as replicates and will be reported with a mean ($\bar{x}$) and standard deviation ($x\sigma_{n-1}$) in most analyses.

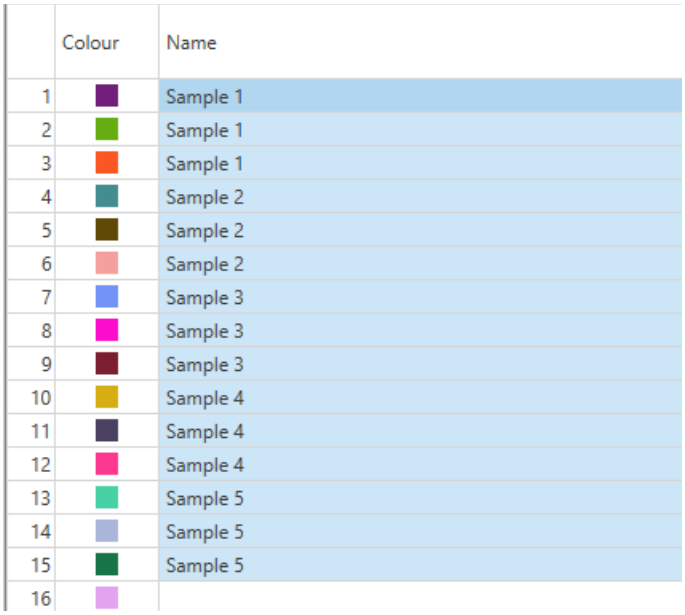
Samples with the same characters but different assays will be linked based on the type of analysis chosen. For example, in *Relative Quantification* a sample with the same characters will be used for both the gene of interest and reference gene. *Cycling Data* from the two independent assays will be linked together to calculate the gene expression value.

You can highlight multiple cells within a column and enter the same characters to annotate replicates. Alternatively, enter the name in one cell, highlight that cell and other cells that will be part of the replicates (use Ctrl + Click to highlight non-adjacent cells), and then select the *Fill down* icon  to give all the selected cells the same name.

Use the Auto fill icon to annotate sequential characters (e.g. sample 1, sample 2, sample 3…). To allow for replicates follow the following process:

Enter the first set of characters for the first name (Sample 1).	
Leave the same number of rows blank as the number of replicates required below the first name. Enter the second name of the sequence (Sample 2).	
Now highlight all the cells required to complete the filling of the names and replicates.	
Click on the Auto fill icon.	

The names will be sequential based on the first two inputs and the replicates for each will be automatically filled in too.



	Colour	Name
1		Sample 1
2		Sample 1
3		Sample 1
4		Sample 2
5		Sample 2
6		Sample 2
7		Sample 3
8		Sample 3
9		Sample 3
10		Sample 4
11		Sample 4
12		Sample 4
13		Sample 5
14		Sample 5
15		Sample 5
16		

Type

Select the sample *Type*.
There are eight options to choose from. The type chosen will determine the way in which the sample is utilised during analysis.
To change multiple cells at once, highlight the cells, use the F2 key on your keyboard, and then select from the following options:

Unknown: Any sample that is under investigation.

Standard: A sample of known quantity, used to generate a standard curve from which an unknown sample quantity can be calculated, or used to determine amplification efficiency.

NTC: A sample that contains no template. NTC's are used to monitor for amplicon contamination in the reaction and for the amplification of non-specific amplicons (primer dimers) when using intercalating dye chemistries.

Positive: The sample is known to contain the target of interest. A positive control is used to confirm that the assay is working and helps prevent false negatives.

Negative: The sample is known not to contain the target of interest. A negative control is used to monitor for contamination of the assay and is helpful in preventing false positives. Unlike the NTC, the negative control can contain an internal amplification control template to ensure the PCR is working.

NRT: The sample has not undergone reverse transcription. The NRT control is used to monitor for genomic DNA amplification during RT-qPCR. Only cDNA, derived from mRNA, should be amplified during RT-qPCR when used to determine gene expression values. If an assay is positive for genomic DNA, consider changing the primer design to target intron exon boundaries. If none exist then improve the extraction of RNA and the removal of genomic DNA from the sample, such as using a DNase.

NAC: The sample does not contain any polymerase. As the NAC contains no polymerase it should not produce an amplification signal. Typically, NAC controls are used to monitor for self-hydrolysis of quenched probes, which could result in a false positive signal.

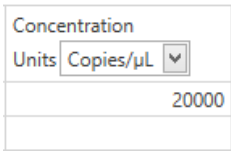
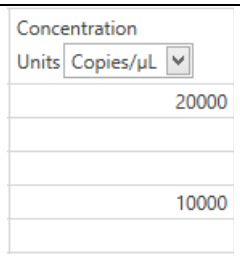
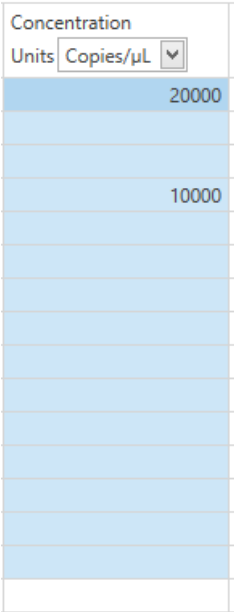
IRC: The same sample is used in two or more different runs. The IRC is used to control for variations between runs and instruments. It is typically applied when multiple runs are used for analysis.

Sample Concentrations

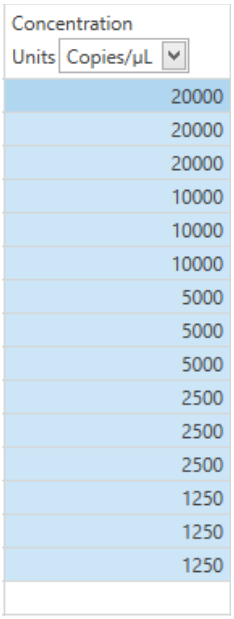
Enter a *Concentration* for each standard.

When using standards it is a requirement to provide a value for each one. The value can be a quantifiable absolute or an arbitrary number. Numbers can also be entered in scientific notation (1E03 = 1 x 10^3 = 1000)

Enter the values one at a time or use the Auto fill option to quickly add a serial dilution and replicates by doing the following:

Enter the first concentration value (20,000).	
Leave the same number of rows blank as the number of replicates required below the first concentration. Enter the second concentration in the dilution series (10,000).	
Now highlight all the cells required to complete the dilution series and replicates.	
Click on the Auto fill icon.	

The concentrations will be filled based on the first two inputs and the replicates for each will be automatically filled in too.

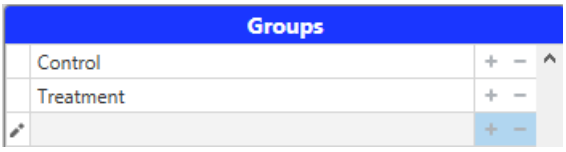


Select the type of units you want to report in.

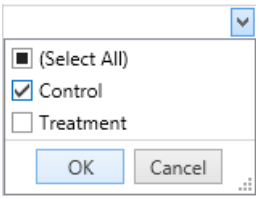
Creating and Allocating Groups

Use sample groups to allow you to calculate statistics for a collection of samples that are not replicates (e.g. Treatment or Control). You will be required to create and allocate groups when using *Relative Quantification* analysis.

Type the group name in the *Groups* table.
Remove groups by using the *Delete* button on your keyboard ensuring that you select the first column in the group row.



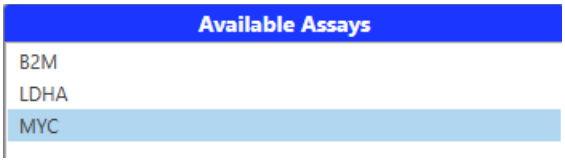
Allocate one or more groups to a sample.
Select the required samples by highlighting the cells in the *Groups* or *Name* column. Use Ctrl + Click to highlight non-adjacent samples. Select the plus button next to the group name to allocate the group. Or click on the selected cells in the *Groups* column and use the drop down list to select the required group(s) by ticking the box next to the group name, or *Select All*, and then click the *OK* button.
To remove a group form the *Group* column, use the delete key of your keyboard, the minus button next to the group name, or click on the cell to bring up the drop down list. Then simply un-check the group from the list provided to remove it, or *Select All* to remove every group.



Linking an Assay to a Sample

An assay must be linked to a sample to allow the software to recognise and properly analyse the sample. Failure to allocate an assay to a sample will result in the sample not being analysed.

Link one *Assay* to a sample.
Select the required samples by highlighting the cells in the *Assays* column. Use Ctrl + Click to highlight non-adjacent samples.
Select the required assay from the *Available Assays* window then drag and drop the assay into the highlighted cells. Alternatively, click on the selected cells in the *Assays* column and use the drop down list to select the required assays(s) by ticking the box next to the assay name, or *Select All*, and then click the *OK* button.
To remove an assay form the *Assays* column, use the delete key of your keyboard or click on the cell to bring up the drop down list. Then simply un-check the assay from the list provided to remove it, or *Select All* to remove every assay.



Information

Enter the name of the *Operator* (Optional).

Enter any *Notes* about the run (Optional).
Provide enough detail to help you understand the run at a later time.
Details may include the experimental purpose of the run or notes regarding the samples (e.g. tissue type).

During a Run

Once acquisition begins, the raw data is displayed for the channels selected under the *Data* section. The data is updated following each acquisition and the signal scaled to provide the best resolution for a group of samples, which can be, highlighted, or selected or deselected during a run. Analysis of data can be conducted prior to a run completing, if sufficient data is available to achieve meaningful analysis. Once the run has completed the raw data will always be available to view in future.

A *Run Summary* banner will appear as soon as the run commences.

In the *Run Summary* banner, the hold temperature or cycle number is displayed to the left side of the banner next to the name of the instrument performing the run.
A graphic of the *Profile Summary* is also displayed. The section at which the run has progressed through is highlighted in grey.
The *Time Remaining* to completion of the run is displayed to the right side of the *Run Summary* banner.

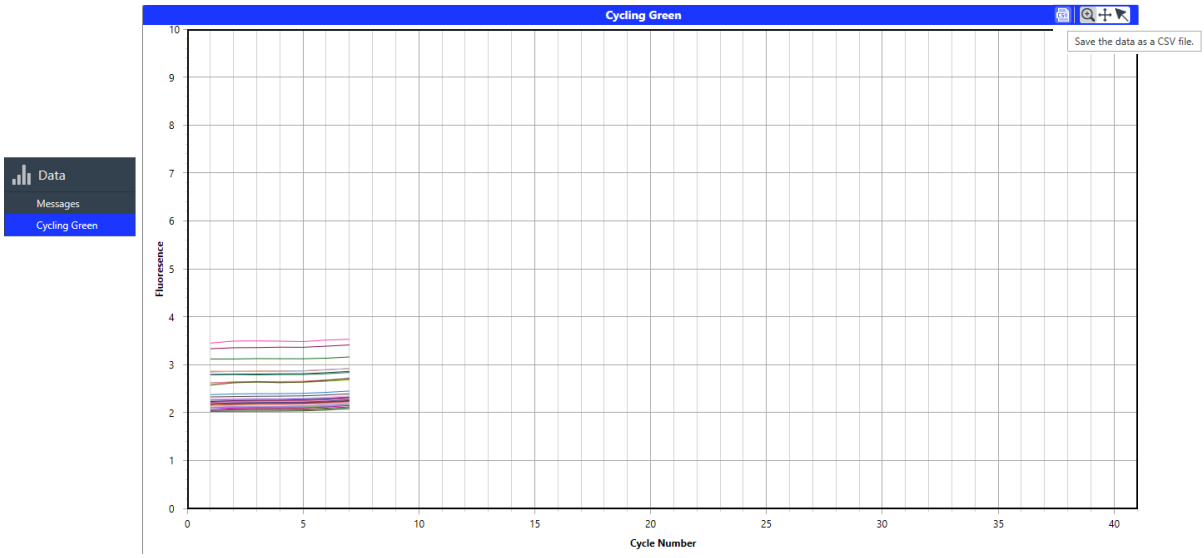
Aborting a Run

You can stop the run at any point by selecting the Abort function in the summary window.

Hot Surface
In the event of a user aborted run do not open the lid until the instrument has cooled. The rotor within the chamber could be above 40°C (104°F). To avoid personal injury, do not touch the rotor for at least 5 min.

Data

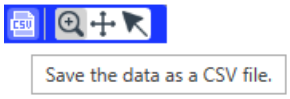
The raw data is displayed for each channel being acquired and is listed in the *Navigator bar*.
Cycle data is plotted as cycle number (x-axis) against the fluorescence value (y-axis) with a maximum fluorescence value of 100 units. At the start of the run the fluorescence will be scaled from 0 – 10 units or 70 – 90 units depending on the *Adjust Gain Settings* option chosen. As the real time curve grows beyond 10 units or drops below 70 units, the graph is auto-scaled to ensure the maximum curve takes up 90% of the visualised graph.



Melt data is plotted as temperature (*x*-axis) against fluorescence (*y*-axis) with a maximum fluorescence value of 100 units. Again the graphs are auto-scaled depending on the *Adjust Gain Settings* option chosen.
To learn more about the various features available on these graphs see XXX.

Export Raw Data

The raw data for *Cycling* and *Melt* can be exported by selecting the *Save the data as a CSV file* icon.



Messages

Any warnings about the run will be displayed in Messages along with the time it occurred.

Common messages will include the start time and instrument name and firmware version. Some messages may be warnings such as incompatibility of a selected assay or any loss in communication with the instrument and when communication was restored.
The *Autogain* values determined during the run will also be reported in the messages.

TimeStamp	Message	Category	Priority
10/15/2015 11:34:46 AM	The assay HBV is not compatible with this run profile.	Run	Warning
10/15/2015 11:34:46 AM	Run started on: mic M0000002 - FW version 1.19	Run	Information
10/15/2015 11:38:31 AM	Autogain completed for Green using Sample: 26, Detector gain: 1x, LED power: 596, Scale: 1.01	Run	Information
10/15/2015 12:30:58 PM	Autogain completed for Green using Sample: 2, Detector gain: 1x, LED power: 750, Scale: 2.04	Run	Information
10/15/2015 12:33:06 PM	Run complete	Run	Information

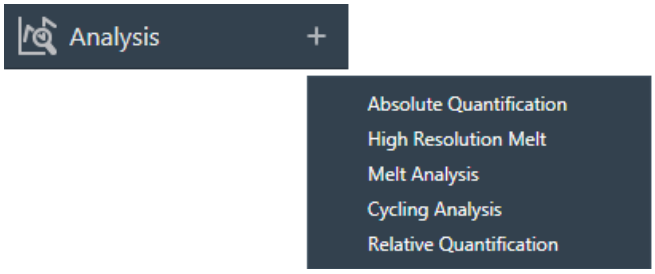
Analysis

There are a number of analysis types available in the software: *Cycling*, *Melt*, *Standard Curve*, *Absolute Quantification*, and *Relative Quantification*. The software also includes three different methods for achieving genotyping analysis, which are dependent on the type of chemistry used; *Melt*, *HRM* or *Allelic Discrimination* (software pending build). Analysis specific parameters are provided for edit, and report tables are displayed along with various graphs depending on the analysis type.

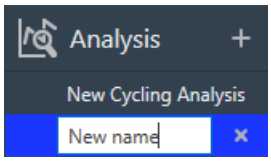
We have taken care to provide the most robust up-to-date methods for qPCR analysis to ensure confidence in the final result. Details regarding some of the analyses are described in the literature and we have provided references to certain publications that best describe these methods.

To start a new analysis, select the *Add* button next to the *Analysis* heading in the navigator bar. Select from the following options:

1. Cycling Analysis
2. Melt Analysis – with Genotyping
3. Absolute Quantification (includes Standard Curve)
4. High Resolution Melting (module version)
5. Relative Quantification



The generic name of the analysis selected will be displayed in the navigator bar. You may edit the name by double-clicking on it.



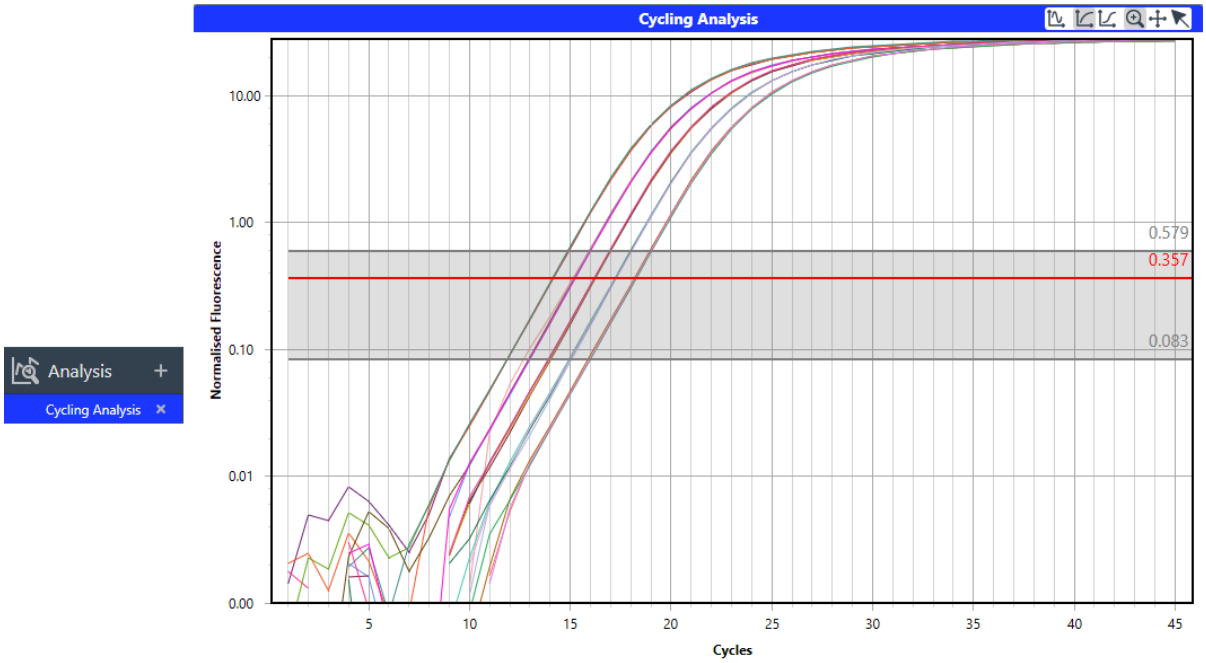
Multiple analyses are possible for each run, with the analysis being viewed highlighted blue on the navigator bar.

Delete any analysis by selecting the *Delete* button next to the analysis name.

Cycling Analysis

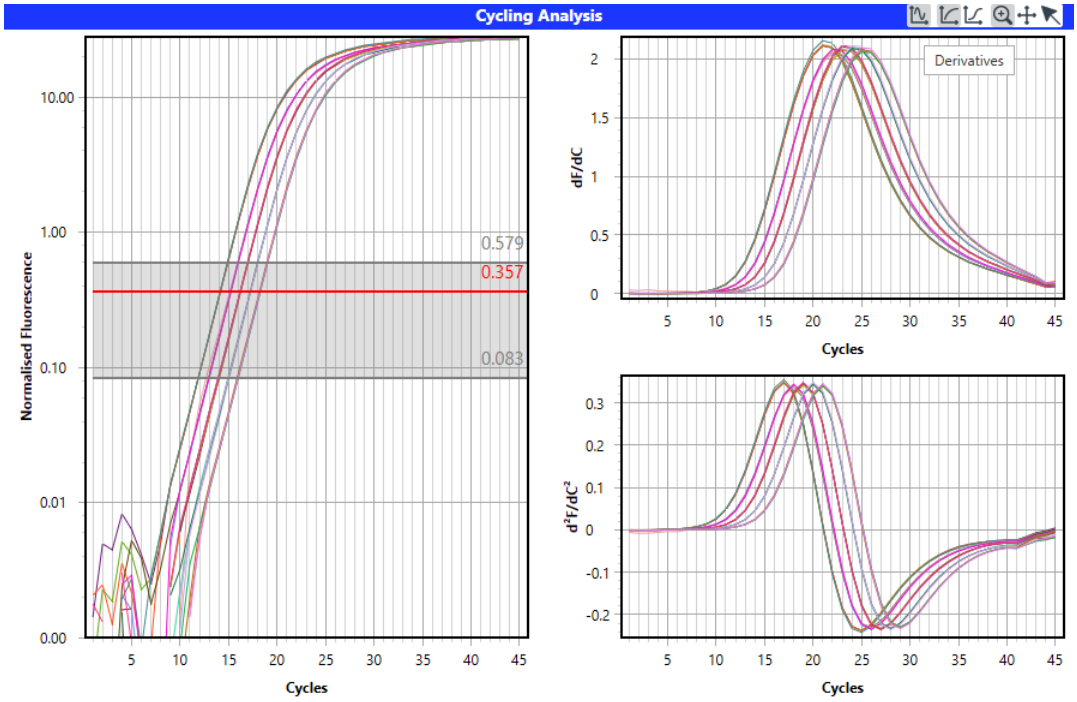
Cycling Analysis allows you to determine the quantification cycle (C_q) and reaction efficiency of each sample in your data set.
Cycling analysis is best used on its own when optimising assays.
Cycling analysis is always provided with any other analysis type that uses cycling data (*Standard Curve*, *Absolute Quantification*, and *Relative Quantification*), allowing you to adjust parameters associated with generating C_q and efficiency values.

By selecting *Cycling Analysis* the software will, by default, plot baseline-corrected curves as fluorescence (*y*-axis) against cycle number (*x*-axis), in logarithmic scale, for the first target that was chosen in the *Assays* list.

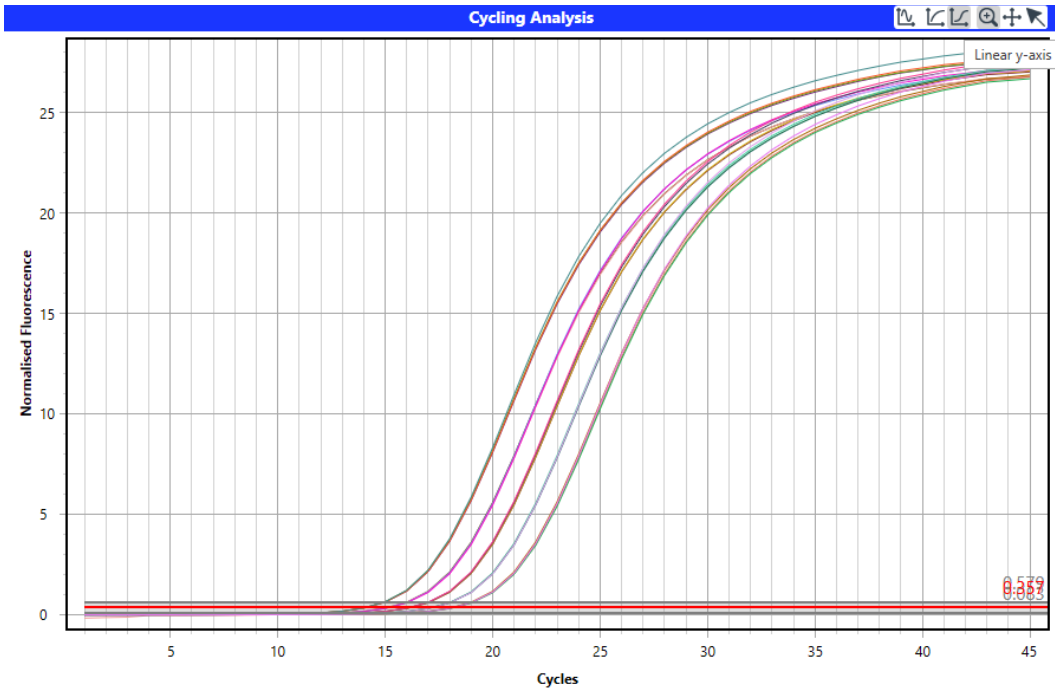


Graph Types

Derivatives: the first and second derivative curves for the selected data can be displayed in parallel with baseline corrected amplification curves. The derivative curves can be used as a reference to set an appropriate threshold or to gauge amplification quality.



Linear *y*-axis: display the baseline corrected cycling data with the *y*-axis in linear scale by selecting the *Linear y-axis* icon.



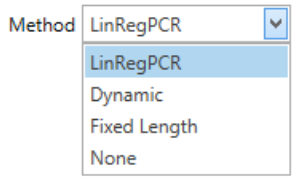
Log *y*-axis: displaying the data in the logarithmic view allows you to better visualise the exponential region of the amplification curve and is therefore the default option. You can revert to the logarithmic scale by selecting the *Log y-axis* icon.

Cycling Analysis Parameters

To change to another target use the *Target drop-down list* in the *Parameters* window.
The channel associated with the target will be displayed next to the target name.

A default set of parameters will be applied to the assay data, providing automatic calculation of C_q and efficiency values, which are reported in the results table. You can change the following parameters:

Method: There are four options available:



LinRegPCR (default): uses a baseline estimation algorithm that iteratively adjusts the baseline to ensure a constant slope is achieved between the upper half of the log linear portion of the amplification curve and the bottom half of the curve for all samples in the assay data set. In effect this reconstructs the exponential phase below the baseline noise. It is based on the assumption that amplification efficiency is constant from the very first cycle onward. This method minimises the propagation of errors in quantification due to errors in efficiency calculation caused by either over or underestimation of the baseline values (Ruijter *et al*. 2009).

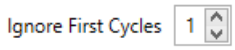
The LinRegPCR algorithm then attempts to set a window of linearity (W-o-L, grey shading) by starting the upper limit of the window at the mean fluorescence level found at the second derivative maximum. Then an iterative modification of the W-o-L is applied until the optimum is found that provides the least variation between individual efficiencies and the mean efficiency of the samples in the data set (Ramakers *et al*. 2003; Ruijter *et al*. 2009). From the W-o-L the *cycle threshold* (red line) is set at 75% of the window region. Samples with no detectable amplification or those below a user defined Fluorescence cutoff will not be included in the W-o-L calculation.
Note: Due to the accumulative nature of signal for hydrolysis probes, LinRegPCR might not be a viable option. The preferred method would be *Dynamic*.

Dynamic: determines the average baseline value measured prior to the detection of specific amplification (take-off), subtracts the average value from the measured values, then takes into account any slope in the baseline curve, to baseline correct the sample. The take-off is calculated by using the second derivative maximum as a starting point. The same algorithm applied in the LinRegPCR method is used to find a W-o-L from which a cycle threshold can be set.

Fixed Length: the baseline average of the first five cycles, after the ignored cycles (see *Ignore first cycles* below), is subtracted from the measured baseline values to baseline correct the sample. This is the most basic method of baseline correction. For some data sets this method may not be compatible with the setting of a W-o-L.

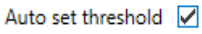
None: no baseline correction. This method allows you to view the raw data in *Cycling Analysis*. A W-o-L is not possible without any baseline correction.

Ignore first cycles: use this setting if there is a significant deviation in the baseline at the start of the run. These changes can occur due to many factors including too much template or insufficient denaturation of double stranded DNA. Applying this may improve analysis using *Dynamic* baseline correction but will have minimal if any effect when using *LinRegPCR*.



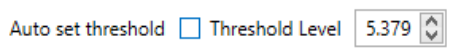
Cycle threshold: The *cycle threshold* is used to determine the C_q value of each selected sample and can be set automatically by the software or manually by the user.

Auto set threshold: uses the W-o-L to set the cycle threshold.
For some data sets the W-o-L may not be determined and the threshold will be set to a default value of 0.01. If this occurs, try using another *Method* or set the threshold manually.
Both the W-o-L and threshold will change if samples are deselected or reinstated into the analysis, as the W-o-L is determined from the assay data set. If you wish to view specific samples without affecting the analysis, then use the *View* function in the *Samples Selector* to remove curves just from the graph rather than the analysis.



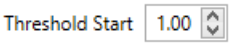
Note: For some data sets with poor amplification, a W-o-L may not be determined, resulting in an inability to set the threshold automatically. Under such circumstances a warning is displayed next to the Auto set threshold field.

Manual threshold: you must deselect the *Auto set threshold* before being allowed to manually set the threshold. Enter the value in the *Threshold level* text box or drag the threshold using your mouse.



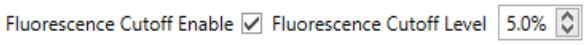
Note: Use the derivative curves and cross hairs to help you set an appropriate threshold. With the cross hairs positioned on the second derivative maximum, reference the same point on the baseline-corrected curves. Set the threshold just below this point to ensure you are within the region of log-linear amplification.

Threshold start: you can avoid interfering parts at the start of the baseline-corrected real time curve by moving the threshold start position. This can be achieved by either entering the value in the *Threshold start* text box or moving your mouse left or right clicked on the green box at the start of the threshold line.



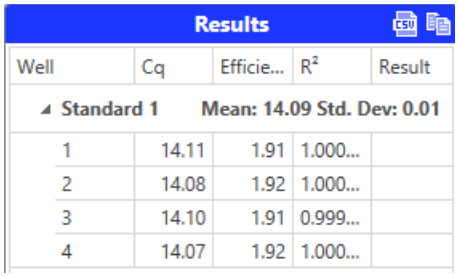
Note: It is not recommended to analyse two different assays using the same cycle threshold or W-o-L. Different assays will have different efficiencies that can affect the performance of the analysis algorithms.

Fluorescence Cutoff: This parameter will exclude samples that fall below a user defined percentage of the maximum fluorescence change. Small changes in fluorescence can interfere with the determination of C_q values by changing the W-o-L. Some of these small changes can be due to probe self-hydrolysis and cross talk between dyes. The default level is set to 5%.



Cycling Analysis Results Table

The results table is organised to display the mean ($\bar{x}$) and standard deviation ($x\sigma_{n-1}$) of the C_q values for sample replicates; and the individual sample results, which are organised, just below the replicate row, into the following columns:



Results

Well	Cq	Efficie...	R^2	Result
Standard 1	Mean: 14.09 Std. Dev: 0.01			
1	14.11	1.91	1.000...	
2	14.08	1.92	1.000...	
3	14.10	1.91	0.999...	
4	14.07	1.92	1.000...	

Well: the order of the well numbers will depend on the grouping of the samples as the name of the sample order can be alphanumeric (grouped) or numeric (ungrouped).

C_q: the quantification cycle value for each sample, which is dependent on the cycle threshold set.

Efficiency: the amplification efficiency is calculated for each sample using the LinRegPCR algorithm described by Ramakers *et al.* (2003). Using the slope of the linear regression line from the calculated W-o-L, efficiency is calculated as; $E = 10^{slope} - 1$.

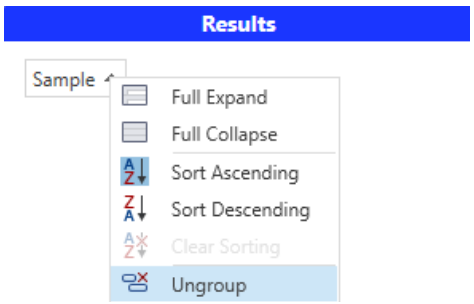
Alternatively, a single efficiency value can be determined for a selected assay using the *Standard Curve Analysis* method.

R^2: the r-squared value is a quality measure of the linear regression used to calculate amplification efficiency (values > 0.98 are acceptable).

Result: any issue related to the quality of the sample is reported in the results column. For example, if a C_q value cannot be determined.

Cycling Analysis Sample Table Selector

The result table can be compressed by hiding the individual sample results using the triangle at the top of each replicate row or selecting the *Full Collapse* option in the *Sample* table selector for the whole table. Right click the Sample table selector to view the different table sorting options.
To sort the samples in replicates using an alphanumeric order use the *Group by this column* option. Otherwise select the *Ungroup* option to have the samples displayed in order of well number.
The individual samples can be sorted in *Ascending* or *Descending* order for any of the columns for both grouped and ungrouped tables. Use this option to determine the range of C_q or efficiency values by displaying the highest to the lowest values.

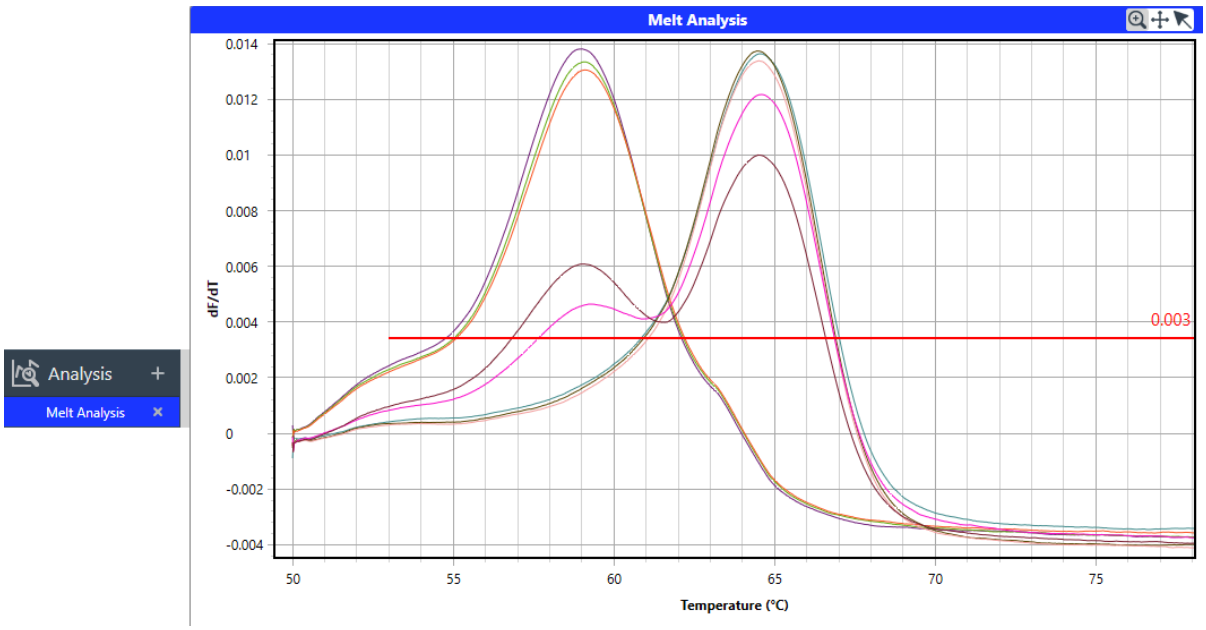


Melt Analysis

Melt Analysis allows you to determine the peak dissociation temperature (T_m) of a sample from the melt data. Basic melt analysis can be typically used as a measure of analytical specificity for an assay, especially when using intercalating dyes, by detecting any non-specific amplicons such as primer dimers. Melt analysis can also be applied for the determination of genotypes using chemistries such as dual hybridisation probes.

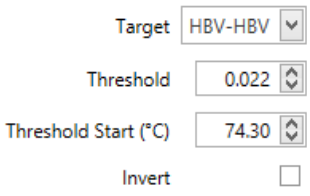
Upon selecting the *Melt Analysis* option a graph will be displayed showing the first derivative curve plotted as $\frac{dF}{dT}$ (y-axis) against temperature (°C, x-axis), for the first target that was chosen in the *Assays* list.

The melt curve threshold can be set to any value, along with various other melting parameters available for genotyping.



Melt Analysis Parameters

To change to another target use the *Target* drop-down list in the *Parameters* window.



Threshold: change the threshold level by either sliding the red line up or down on the graph; or by entering a numerical value in the *Threshold level* text box. Only peaks above the threshold line will be reported.

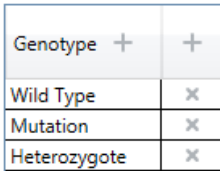
Threshold Start: to ignore earlier peaks, slide the red line from left to right by clicking on the green box at the start of the threshold line, or enter a numerical value in the *Threshold Start Temperature* text box.

Invert: the first derivative melt curves can be inverted to allow for analysis of data generated using chemistries such as quenched FRET dual hybridisation probes or Plexor®. Tick the *Invert* box to invert the melt curves.

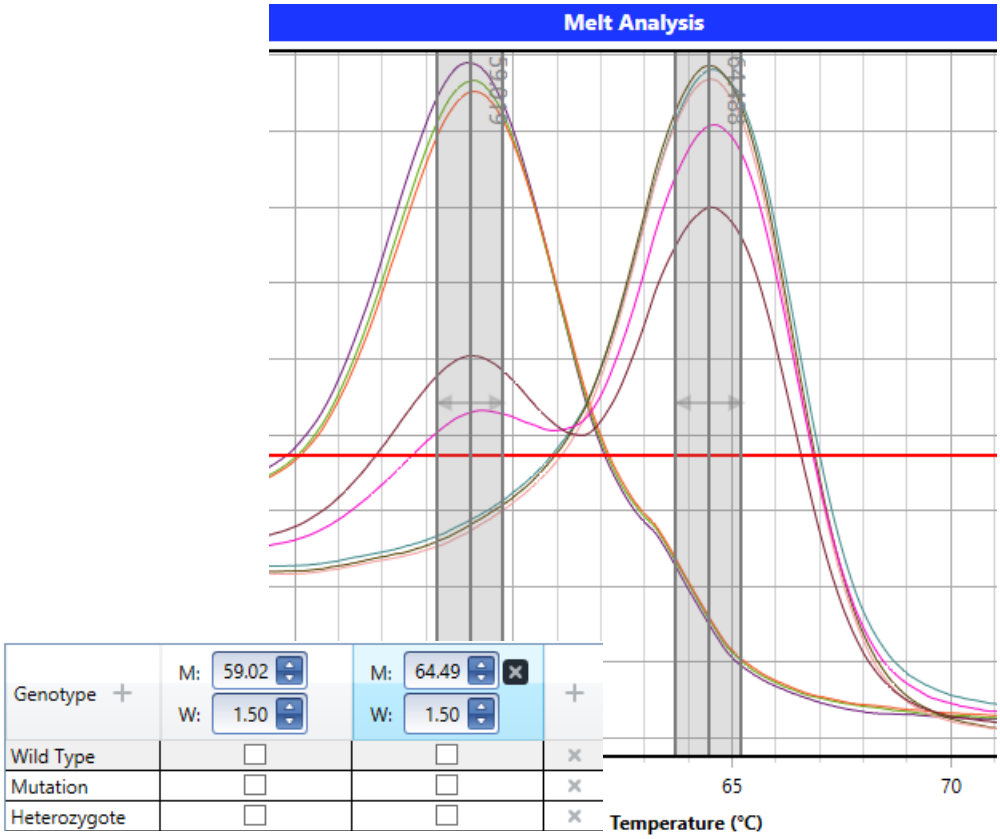
Melt Genotyping

Classify samples into known genotypes by using the specific T_m values associated with each genotype.

Enter a *Genotype* by typing the name in the column provided.
Use the Enter key to add another genotype.

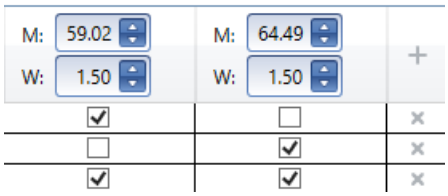


Create T_m *Bins* for each allele.
Enter a name for each *Bin* or keep the default name (Bin 1).
Select the *Peak* T_m for each bin by either moving the centre of the bin left or right, or entering a value in the *Peak* text box provided in the Genotyping table.
Select the *Width* of the bin by moving the outer perimeter inwards or outwards, or entering a value in the *Width* text box provided in the *Genotyping table*. The width allows for variations in T_m between samples. Ensure that the bin widths do no overlap with other bins.
Hovering over the *Bin* text box in the *Genotyping table* will highlight the bin in the melt graph. The *Bin* name will also be displayed within a tab on the left side perimeter of the bin within the graph.

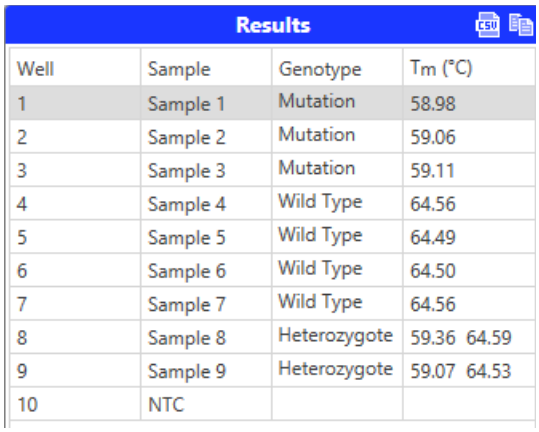


Link the *Genotype* to a *Bin* by using the tick boxes under each bin column within the row for each genotype.

If a genotype shares both bins (heterozygote) then tick both boxes.



Melt Analysis Results Table



Results

Well	Sample	Genotype	Tm (°C)
1	Sample 1	Mutation	58.98
2	Sample 2	Mutation	59.06
3	Sample 3	Mutation	59.11
4	Sample 4	Wild Type	64.56
5	Sample 5	Wild Type	64.49
6	Sample 6	Wild Type	64.50
7	Sample 7	Wild Type	64.56
8	Sample 8	Heterozygote	59.36 64.59
9	Sample 9	Heterozygote	59.07 64.53
10	NTC		

Melt temperature peaks (T_m) are displayed in the *Results* table.
Samples with peaks below the threshold line will be reported as '*No melt temperature*'.

Genotypes will be reported in the *Genotype* column if the *Genotypes table* has been edited.

Samples can be excluded from analysis or removed form view in the graph by using the *Samples selector*.

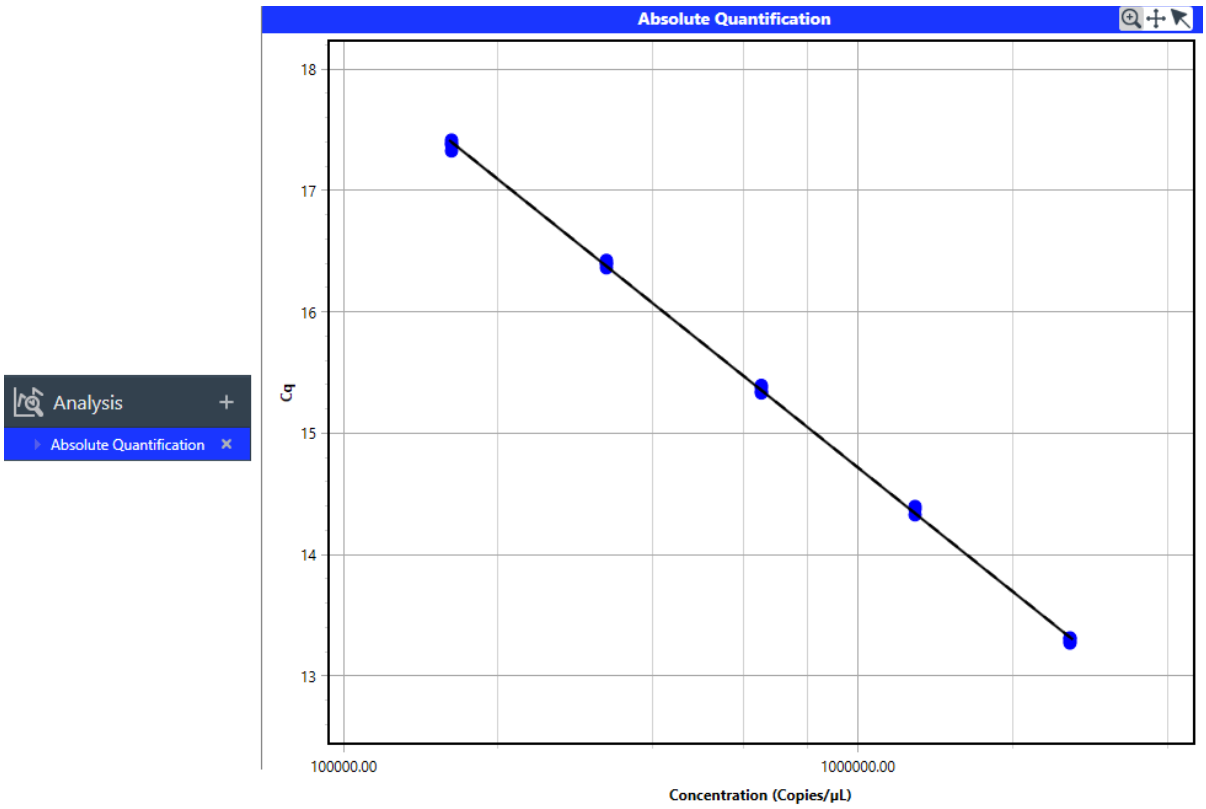
Display the samples according to sample name in ascending or descending order (alphanumeric) by clicking on the *Sample column* heading. To display the samples in order of well number (ascending or descending) select the *Well column* heading. To display samples in the order of highest to lowest or lowest to highest T_m values select the T_m (°C) column heading.

Standard Curve Analysis

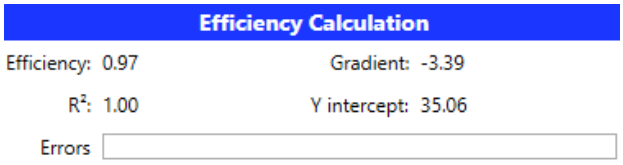
The *Standard Curve Analysis* feature allows you to determine the efficiency of an assay by using a serial dilution series of known sample. This method can be used as an alternative to the LinRegPCR method in calculating reaction efficiency.

Efficiency Calculation

The method utilises the *Cycling Analysis* feature to determine C_q values and plots them (y-axis) against the log of the given concentration (x-axis) for each standard annotated in the *Samples editor*. A line of best fit is generated for the data plot from which the slope of the line is determined.



From the slope of the line the efficiency is calculated using the equation $10^{\frac{-1}{Slope}} - 1$, and is reported in a summary window as a value from 0 to 1 along with the r-squared (R^2) value, gradient of the line (M), and y-intercept.



You can also enter the efficiency value for the assay into the *Relative Quantification* analysis option in the *Assay setup*. This value will be used in future relative quantification analyses as the default efficiency.

R-squared Value: The R^2 value is a measure of the percentage of data that matches the hypothesis that the given standards form a standard curve. In other words if the R^2 value is low then the given standards do not aggregate to the line of best fit very well, and therefore, the calculated efficiency may not be reliable**.**

A value > 0.98 is typically a good R^2 value. However, a good R^2 value can still be achieved for a poor standard curve if not enough standards have been used. It is recommended that the standards extend to at least 5 $\log_{10}$ concentrations.

Standard Curve Analysis Parameters

To change to another target use the *Target* drop-down list in the *Parameters* window.

Standard Curve Results Table

The *Standard Curve Results table* contains the following measures:

C_q: the quantification cycle value for an individual standard.

Given Concentration: the given concentration is the value annotated in the *Samples editor*. The reported units are also displayed in the column heading. The unit measure is selected from a list in the *Samples editor*.

Calculated Concentration: is an adjusted concentration for an individual standard based on the line of best fit. The adjusted concentration is calculated by using the C_q value to interpolate the new concentration from the line of best fit. The geometric mean $(\prod_{n=1}^{k} x_n)^{\frac{1}{k}}$ for a set of replicates is also provided in the top row for the set.

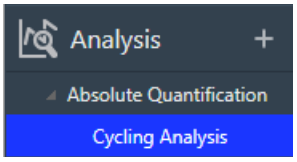
Percentage Variation: is the percentage difference between the given and calculated concentrations.

Standard Curve Results

Well	Sample	Cq	Given Concentration (Copies/µL)	Calculated Concentration (Copies/µL)	% Variation
1	Standard 1	14.11	2600000.00	2608230.44	0.32
2	Standard 1	14.08	2600000.00	2648598.47	1.87
3	Standard 1	14.10	2600000.00	2615842.49	0.61
4	Standard 1	14.07	2600000.00	2671806.22	2.76
5	Standard 2	15.12	1300000.00	1308950.27	0.69
7	Standard 2	15.16	1300000.00	1271201.54	2.22
8	Standard 2	15.18	1300000.00	1256407.62	3.35
9	Standard 3	16.18	650000.00	636175.37	2.13
10	Standard 3	16.16	650000.00	643828.86	0.95
12	Standard 3	16.12	650000.00	660152.89	1.56
13	Standard 4	17.15	325000.00	328199.29	0.98

Cycling Analysis for Standard Curves

Cycling Analysis is used to determine the C_q values for the standard curve. Therefore, *Cycling Analysis* is automatically paired with, and will appear beneath, the *Standard Curve Analysis* in the navigator bar. Use *Cycling Analysis* to make modifications to analysis parameters such as *Method* or *Ignore first cycles*. The same assay parameters as defaulted for an individual *Cycling Analysis* will be applied when paired with *Standard Curve Analysis*.
Using the *Samples selector* to remove samples from the *Cycling Analysis*, will result in a recalculation of the amplification efficiency in the Standard Curve Analysis.

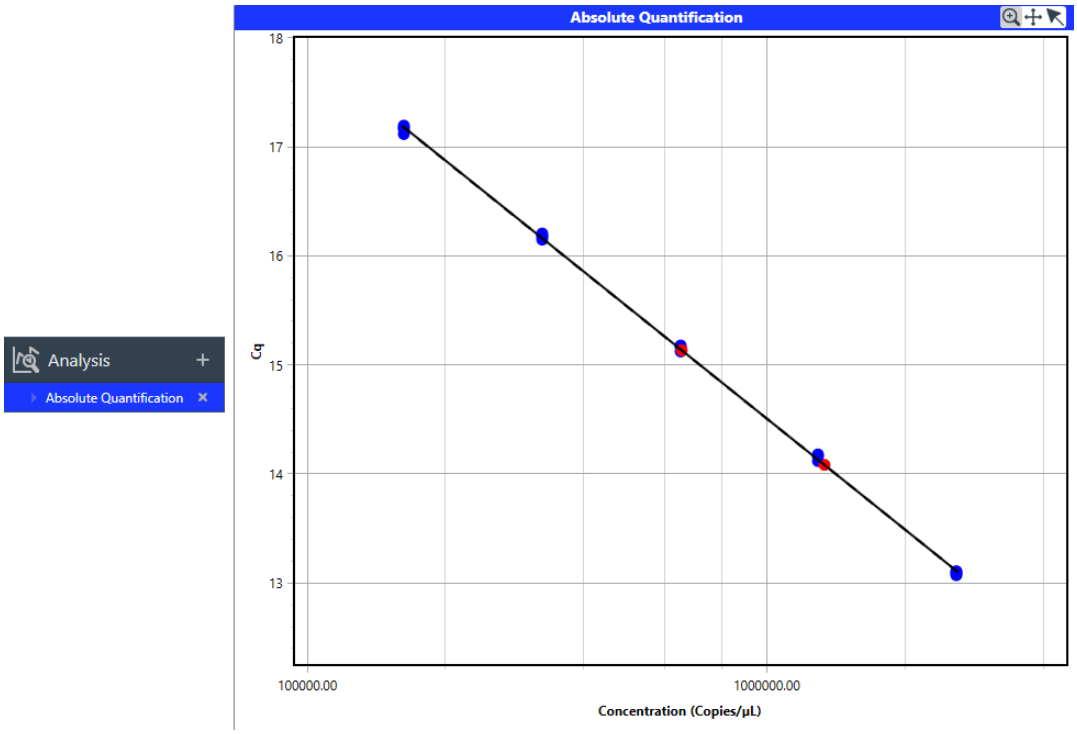


Absolute Quantification

Absolute Quantification allows you to quantify the unknown concentration of a sample using a standard curve generated with samples of a known quantity. The method has been applied to applications such as the determination of viral load in patients.

The *Absolute Quantification* analysis method shares all of the features found in *Standard Curve Analysis* but with the addition of determining unknown concentrations.

In the graph for the standard curve, standards are shown as ***blue*** dots and unknowns as ***red*** dots. Samples that fit outside the limits of the standard curve will be displayed on a dashed line. Samples outside the limits of a standard curve should be treated with caution. To avoid having samples outside the limits of the standard curve, ensure that your standard curve contains enough points to encompass all of your potential unknowns. This may require the determination of the linear dynamic range and/or limit of detection (LoD) for the assay.



Sample Results Table

The *Sample Results table* reports the *Calculated Concentration* for each unknown sample. The geometric mean $(\prod_{n=1}^{k} x_n)^{\frac{1}{k}}$ for a set of replicates is also provided in the top row for the set. The reported units are displayed under the *Calculated Concentration* title and are chosen from a list in the *Samples editor*.

The C_q values for each sample are also reported in the *Sample Results table*.

Sample Results

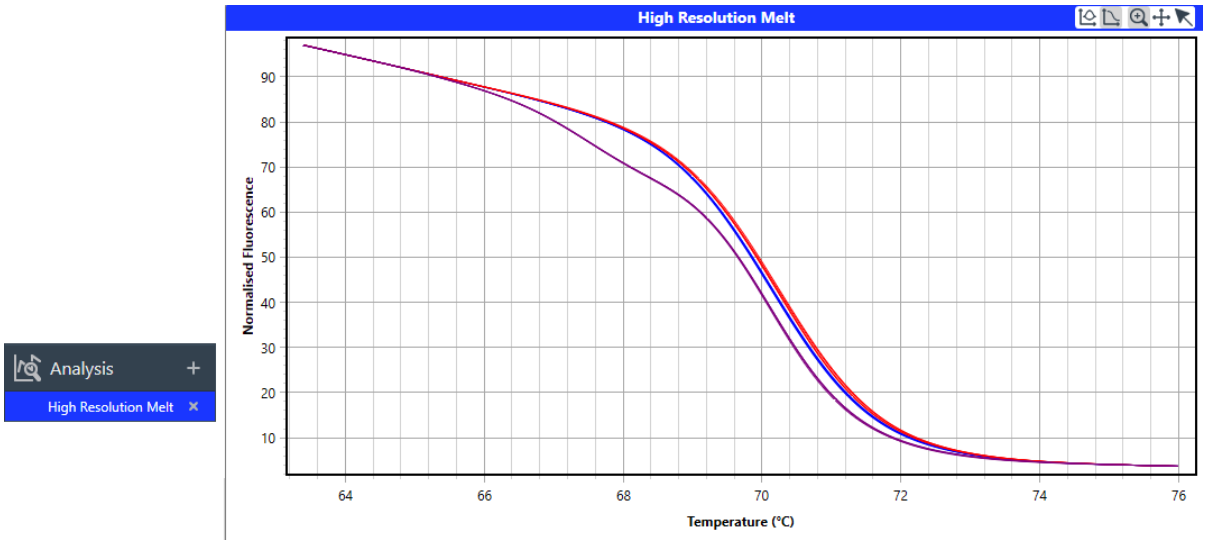
Well	Sample	Cq	Calculated Concentration (Copies/µL)
6	Unknown Sample 1	15.08	1341174.36
11	Unknown Sample 2	16.13	655176.83

High Resolution Melt Analysis

HRM will allow you identify DNA sequence variants, including single base changes, insertion-deletions, and base pair substitutions by analysing DNA melt curves. The software characterizes DNA samples according to their dissociation behaviour as they transition from double stranded DNA to single stranded DNA with increasing temperature.

HRM analysis is provided as an additional software module that will need to be activated using a key, provided upon sale of the software (see Mic Icon page 21).

Selecting the *High Resolution Melt* analysis option will bring up a graph with the normalised melt curves for all samples selected for the first target that was chosen in the *Assays* list. Normalisation is dependent on the regions set in the HRM parameters. The determination of genotypes can only be achieved once the reference genotypes have been edited in the HRM parameters.



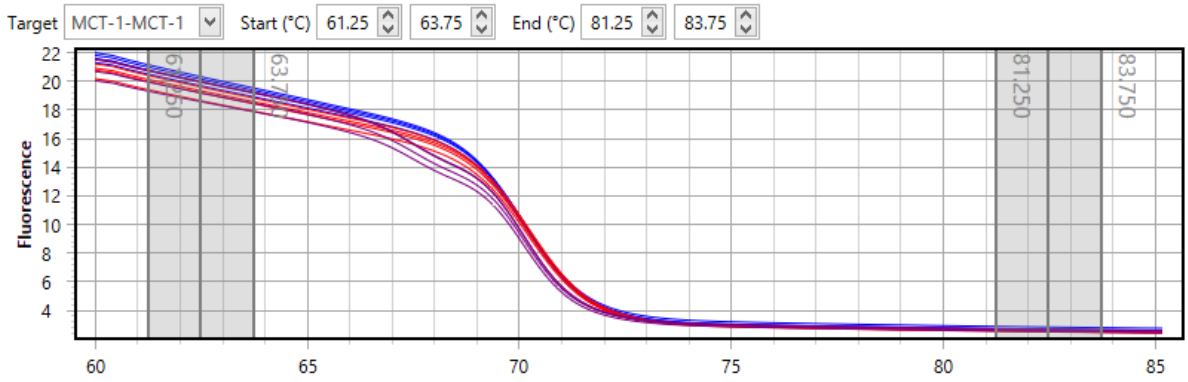
HRM Analysis Parameters

To change to another target use the *Target* drop-down list in the *Parameters* window.

Normalisation Regions

Set the normalisation regions for the selected assay on the raw data plot.
Use the *Raw melt* curve graph to set the two normalisation regions by dragging the midpoint line left or right for each region. Move the ends to widen or narrow the normalisation regions. Alternatively, enter the values for the *Start* and *End* regions in the text boxes provided. The software will prevent the *Start* and *End* regions from crossing over. Data outside of the regions is ignored.

Ensure that the regions encapsulate the part of the melt curve associated with the characterisation of the assay genotypes. Avoid setting the normalisation regions in areas containing large transitions in fluorescence as this may adversely affect the normalisation algorithm.



Reference Genotypes

Reference genotypes will be used to classify each unknown sample into a specified genotype by comparing and matching the unknown curve to the nearest curve of each of the reference genotype curves.

Reference Genotypes

Well	Name
1: Wild Type Standard	Wild type
5: Mutation Standard	Mutation
9: Heterozygote Standard	Heterozygote

Create a new reference genotype by editing the *Reference Genotypes table*.
Select the sample you wish to use as the genotype reference by using the drop down list in the *Well* column. Then enter the *Name* of the reference genotype in the second column.
You can delete any reference genotype by using the delete key on your keyboard.
Enter as many reference genotypes as required for the analysis. This may include allele percentages for more complex studies such as somatic mutations or DNA methylation.

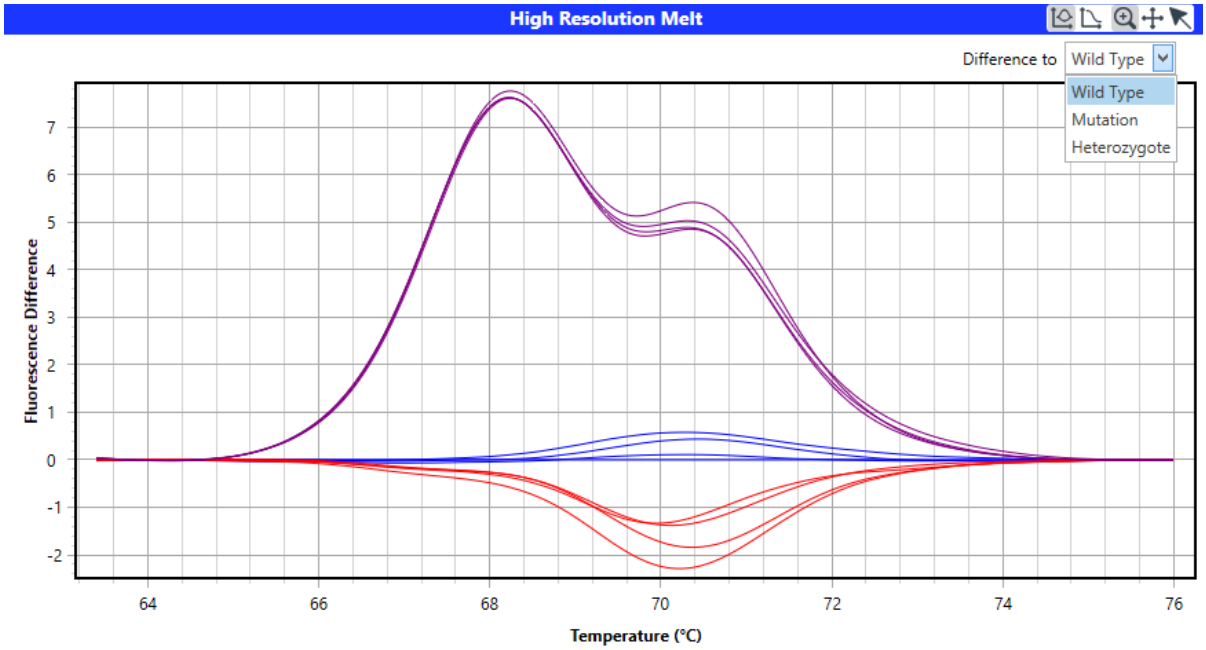
Once the reference genotypes have been set, the software will automatically classify each unknown to a genotype.

Confidence Threshold: A percentage *Confidence Threshold* can be entered and used as an integrity check of the auto-called results. The confidence threshold ensures samples that do not match closely to one of the reference genotypes can be flagged in the analysis.

Difference Plots

Once the reference genotypes have been set, the *Difference Plot* option can be used. The difference plot will display the differences for all analysed curves to a selected reference genotype (this is typically the wild type but can be any of the reference genotypes set). This graphical plot will allow for a better visualisation of curve character differences between genotypes.

Difference plots can be displayed on the main graph of the HRM analysis by selecting the *Difference Plot* icon, then selecting the reference genotype to compare to, from a drop down list.
To revert to the standard normalised curves select the *Normalised Curve* icon.



HRM Analysis Results Table

HRM analysis results are reported as a genotype along with a confidence percentage. The closer the confidence percentage is to 1, the closer the match of the unknown to the reference genotype. Samples that fall below a confidence percentage are reported as an *Unknown genotype*.
Samples with low percentages could be due to poor amplification and should be reanalysed, or investigated further for the potential of additional variations using sequencing methods.

Results

Well	Sample	Genotype	Confidence %
1	Wild Type Standard	WT	100.00
2	Sample 1	WT	97.45
3	Sample 2	WT	99.79
4	Sample 3	WT	94.83
5	Mutation Standard	MUT	100.00
6	Sample 4	MUT	99.60
7	Sample 5	MUT	86.62
8	Sample 6	MUT	94.17
9	Heterozygote Standard	HET	100.00
10	Sample 7	HET	95.73
11	Sample 8	HET	96.23
12	Sample 9	HET	97.73

Relative Quantification

Relative quantification allows you to analyse differences in gene expression for a given sample group relative to a control group; for example, measuring gene expression in response to a drug. To achieve relative quantification an endogenous reference gene must also be run in parallel with the gene of interest to normalise for variations in sample loading. According to the MIQE guidelines, two or more reference genes are required to ensure validity in the normalisation[5, 6]. Furthermore, the efficiency of each gene must be taken into consideration when calculating expression values.

The software uses the relative expression software tool (REST©, Pfaffl et al. 2002) to calculate gene expression ratios between two groups. The method also provides statistical analysis of the calculated expression values. REST uses a mathematical model for calculating gene expression ratios that takes the individual efficiency of each target (gene of interest and reference gene) into consideration (Pfaffl, 2001). The following formula is used to calculate a relative expression value between two groups:

$$ratio = \frac{(E_{gene\ of\ ineterest})^{\Delta Cq_{gene\ of\ interest}(control-treatment)}}{(E_{Reference})^{\Delta Cq_{reference}(control-treatment)}}$$

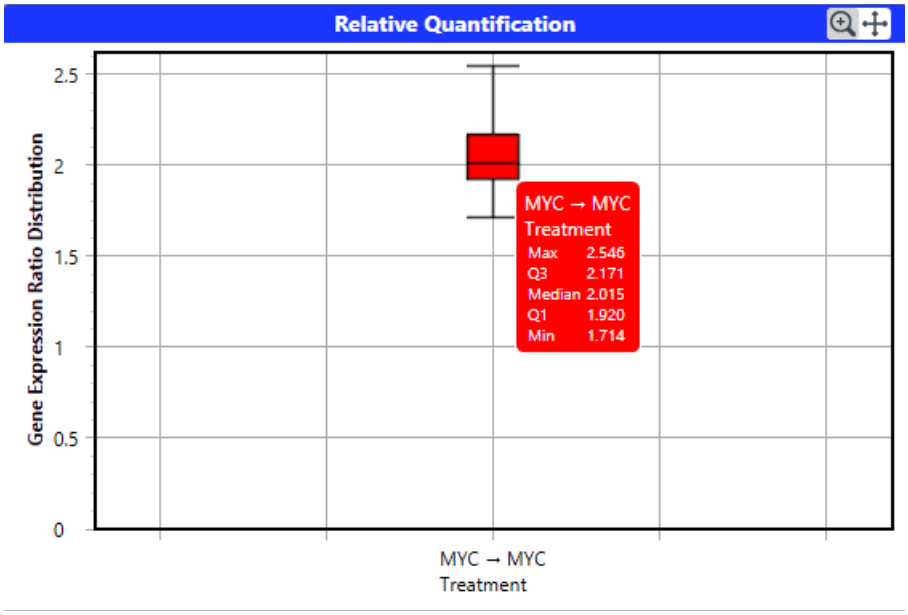
Where, E = reaction efficiency, Cq = cycle threshold value; control and treatment are the two groups. A modification to the formula allows the use of two reference genes for normalisation.

The Cq values are determined using cycling analysis, with LinRegPCR being the preferred method when using intercalating dyes. Efficiency values can be calculated using the same method or using a standard curve.

By using randomisation and bootstrapping techniques, REST allows for a statistical evaluation of the calculated expression ratios. Unlike other statistical methods such as a student t-test, the techniques used in REST do not assume normal distribution in the data, which cannot be expected every time when using ratios.

Gene expression ratios are graphed as *Box and Whisker plots*, where the box represents the upper and lower quartiles, with a median line, and the minimum and maximum observations are the whiskers. If you hover over the box a tool tip will appear with the numerical values for box and whisker plot. Different genes and groups are presented on the same graph with the default minimum range being 0 to 2 fold. You can use the *zoom* and *pan* functions to make closer observations of the graphs.

[5] The use of a single reference gene must be justified by clear evidence that the expression level does not change under the experimental conditions applied.
[6] micPCR software does not yet apply algorithms to determine the stability of reference genes.



Relative Quantification Analysis Parameters

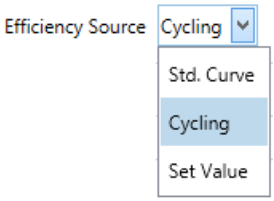
To achieve Relative Quantification, the Sample Editor must be annotated such that each sample is linked to an assay Target and Group. The following parameters can be edited:

Efficiency Source: select the method of calculating efficiency.

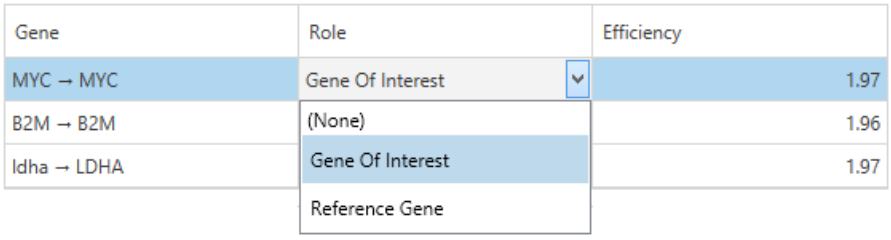
Standard Curve: uses a Standard Curve analysis to determine the reaction efficiency. If this option is selected then the software will provide a daughter analysis below the relative quantification analysis, to allow the user to view and change parameters associated with generating the standard curve. Ensure the standard curve is annotated correctly in the *Samples editor*.

Cycling: the mean efficiency is determined using the LinRegPCR calculated efficiencies for each sample of a target.

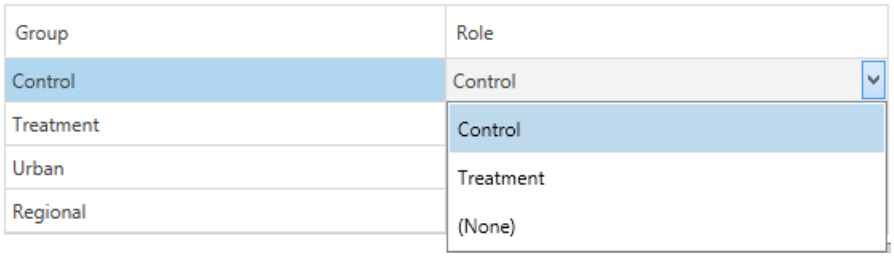
Set Value: enter the efficiency value for each target manually. This value can be stored for each target in the *Assay setup*.



Gene Role: each *Assay Target* used in the run must be selected as a *Gene of Interest*, a *Reference Gene*, or *None* (not used in analysis). The roles can be defined and stored in the *Assay setup*. The efficiency being used is displayed next to the gene role. Multiple genes can be used per analysis.



Group Roles: characterise each group as a *Control*, *Treatment*, or *None* (not used in calculation). Multiple groups can be used per analysis.



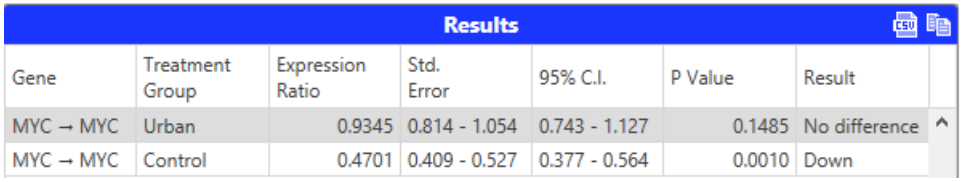
Relative Quantification Analysis Results Table

Gene: the gene(s) of interest being investigated.

Treatment Group: selected treatment group(s) being compared with a control group.

Expression Ratio: difference in gene expression between two groups.

Standard Error and 95% C.I.: Expression level confidence intervals at 68% (equivalent to a single standard error) and 95% are calculated using the bootstrapping technique.

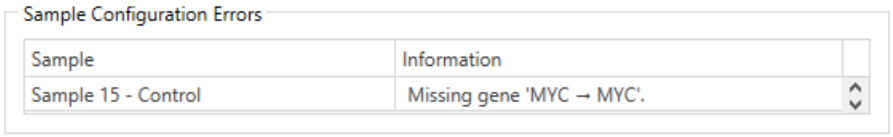


Gene	Treatment Group	Expression Ratio	Std. Error	95% C.I.	P Value	Result
MYC → MYC	Urban	0.9345	0.814 - 1.054	0.743 - 1.127	0.1485	No difference
MYC → MYC	Control	0.4701	0.409 - 0.527	0.377 - 0.564	0.0010	Down

P Value: The hypothesis test P(H1) gives the probability that any observed difference between the two groups is due to chance. This is achieved using up to 10,000 random reallocations of control and treatment samples between the groups, and determines the number of times the expression values for the random assigned groups is greater than the sample mean. Expression values that have a P(H1) < 0.05 are considered to be statistically significant.

Result: text annotation of gene expression result described as down, up or no difference.

Error messages: if there are missing bits of data (e.g. missing reference gene C_q values for a sample), the software will report the error along with which sample, group, or target is missing.



Cycling Analysis for Relative Quantification

Cycling Analysis is used to determine the C_q values for the relative quantification calculations. Therefore, *Cycling Analysis* is automatically paired with, and will appear beneath, the *Relative Quantification Analysis* in the navigator bar. Use *Cycling Analysis* to make modifications to analysis parameters such as *Method* or *Ignore first cycles*. The same assay parameters as defaulted for an individual *Cycling Analysis* will be applied when paired with *Relative Quantification Analysis*.

Using the *Samples selector* to remove samples from the *Cycling Analysis*, will result in a recalculation of the expression ratios. Unlike the other analyses, the samples in the *Samples selector* for *Relative Quantification* are displayed for a group of *Targets* rather than each individual well. This ensures consistency in turning samples on and off.

Appendix A

Toshiba Bluetooth® Work Around

If you are using a Toshiba computer and you are not able to communicate with the Mic instrument using Bluetooth the following work around should rectify the issue.

1. Open the Device Manager.

In Windows® 8 point to the lower-right corner of the screen, move the mouse pointer up, and then click *Search*
Enter Device Manager in the search box, and tap or click Device Manager. You might be asked for an admin password or to confirm your choice.

In Windows® 7, click on the windows start button;
Select the control panel option;
Click on the "Hardware and Sound" heading;
Under the "Devices and Printers" heading, click on the "Device Manager".

2. Open the properties dialog for your Bluetooth adapter.

Select the Bluetooth section header and a number of child items will appear.

In Windows® 8, select the Bluetooth VX Module (where X is a version number) as the Bluetooth adapter.

In Windows® 7, select the "Bluetooth USB controller" device as the Bluetooth adapter.

If this device is not present then you are probably not using the Toshiba Bluetooth drivers. You should not progress further with this trouble shooting process

3. Right click on the Bluetooth Adapter and select the "Update Driver Software" option.

4. Select the "Browse my computer for drive software" option.

5. Select the "Let me pick from a list of device drivers on my computer" option.

6. Select the "Generic Bluetooth Adapter" option.

7. Click on *Next*. Windows® will install the selected driver.

8. Windows® may ask to restart the computer. Before restarting, ensure all your data in all your applications is saved.

9. Restart the micPCR application. The Bluetooth devices should now be discovered

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Abbreviations

CI: Confidence interval

C_q: Quantification cycle

CV: Coefficient of variation

FRET: Forster Resonance Energy Transfer

HRM: High resolution melting

IAC: Internal amplification control

IRC: Internal run control

LoD: Limit of detection

NAC: No amplification control

NRT: No reverse transcriptase

NTC: No template control

PCR: Polymerase chain reaction

qPCR: Real time PCR

RIN: RNA integrity number

RT-qPCR: Reverse transcription real time PCR

SD: Standard deviation

T_m: Melting temperature

UDG: Uracil DNA glycosylase

W-o-L: Window of linearity

Glossary

Analytical Accuracy: the difference between the experimentally measured and actual concentrations.

Analytical Repeatability: precision of the assay within the same samples repeatedly measured in the same assay. Also referred to intra-assay variance it is expressed as the SD for the C_q variance or CV of the copy number/ concentration variance.

Analytical Reproducibility: the variation in results between runs or different laboratories. It is also referred to as inter-assay variance and is expressed as the SD or CV of copy number or concentration. As C_q's typically vary between runs the reporting of inter run variation is not appropriate.

Analytical sensitivity: the minimum number of copies in a sample that can be measured accurately with an assay.

Analytical specificity: assay detecting the specific target sequence rather than another, nonspecific, target. Use of NTC's helps determine analytical specificity.

Dual hybridisation probes: rely on FRET of a reporter dye, attached to the 3' end of one probe (donor), to a quencher molecule, attached to the 5' end of a second probe (acceptor). When the two probes are hybridised adjacently to each other, energy transfer results in a reduction in the reporter dye fluorescence due to the close proximity of the quencher molecule. Another iteration of dual hybridisation probes uses a dye as the acceptor, resulting in an increase in the emission signal of the acceptor dye due to the FRET.

Hydrolysis probes: are short oligonucleotides with a fluorescence reporter dye at the 5' end and a quencher molecule at the 3' end. When the probe is intact, the close proximity of the reporter dye to the quencher results in little fluorescence being detected. During extension the polymerase will cleave the probe through exonuclease activity separating the reporter dye from the quencher, resulting in increased fluorescence.

Intercalating dye: intercalating dyes, such as SYBR® Green I, bind to double stranded DNA. The unbound dye exhibits little fluorescence in solution, but upon binding to double stranded DNA, the fluorescence is enhanced.

Limit of Detection (LoD): the LoD is the minimum concentration that can be detected with reasonable certainty (typically 95% probability).

Linear dynamic range: is the highest to the lowest quantifiable copy number determined by means of a standard curve.

Magnetic induction: when a conductor such as a metal is exposed to a magnetic field it produces a circular electric current, also known as an eddy current, in the conductor. Due to the resistance of the conductor it becomes hot. In the MIC instrument the rotor is the conductor that becomes hot under as it is exposed to a magnetic field.

Quenched FRET: occurs when an excited dye, in close proximity to a quencher molecule, transfers its emission energy to the quencher molecule, resulting in a reduction in signal from the reporter dye.

Reference gene: are genes that are stably expressed within the experimental samples and are used to normalise for variations in extraction yield and reverse-transcription yield.