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mic pcr

Mic Real Time PCR Cycler
User Manual

Version 2.12 Rev C

Revision History

Revision Number	Revised On	Revision Description
2.10 Rev B	31/08/2021	Thermal specifications updated.
2.12 Rev A	18/09/2021	LIMS and V-cap specifications added.
2.12 Rev C	11/11/2021	Mic Rapid Cap tool and additional consumables added.

Contents

Disclaimer

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

The purchase of this product with HRM includes a limited, non-transferable license for all fields other than human or veterinary in vitro diagnostics under specific claims of U.S. Patent Nos. 6,174,670; 6,569,627; 7,387,887; 7,582,429; 7,670,832; 7,803,551; 8,068,992; 9,093,002; 8,296,074; and 9,273,346, owned by the University of Utah Research Foundation and licensed to BioFire Defense, LLC and Roche Diagnostics GmbH.

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

NOTE   A support package should be provided with all questions related to software issues. See Help Icon on how to generate a support package. Please avoid sending screen captures as they have limited information about the issue.

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 is 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 **Power Supply Grounding**
Power supply must be connected to an outlet with appropriate grounding means.

WARNING **Main Supply Cord**
Do not replace detachable main supply cord with an inadequately rated cord.

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 Tube Clamp**
Ensure that the magnetic tube clamp 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.

CAUTION

⚠️ **Authorised Service Only**
There are no user serviceable parts inside the instrument. Service should only be performed by an authorised party.

CAUTION

⚠️ **Power Disconnection**
The power cord set is to be used as a means of power disconnect. Pull the equipment power cord in case of emergency.

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.

Contains FCCID: S7AIW03 Bluetooth® module, which can be found at the back of the instrument when the covers are removed.

UL Listing
UL has tested representative samples of the product and determined that it meets UL's requirements for Laboratory Equipment.

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].

BIOLOGICAL HAZARD

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 support@biomolecularsystems.com.

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[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. Once multiple runs are generated, they can be combined into a single analysis of up to 10 runs for a total of 480 samples. 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.

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[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 *In*formation for Publication of *Q*uantitative Real-Time PCR *Ex*periments (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	± 0.1°C (MIC-2 and MIC-4 models)
		± 0.05°C (MIC-4+HRM model only)
	Ramp Rates	Heating: 4°C/s (fast mode)
		Cooling: 3°C/s (fast mode)
	Temperature Input Range	35 – 99°C (min 40°C when cycling)
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	5 – 30 µL (Standard Caps)
		5 – 25 µL (V-caps[5])
Operating Environment	Temperature	18 – 30°C
	Relative Humidity	20 – 80%

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[5] V-caps are only used with Mic Automation units

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	LED indicator	When illuminated blue, indicates that the instrument is powered 'On' or green, the instrument is 'Running'.
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.

Top

Front   Back

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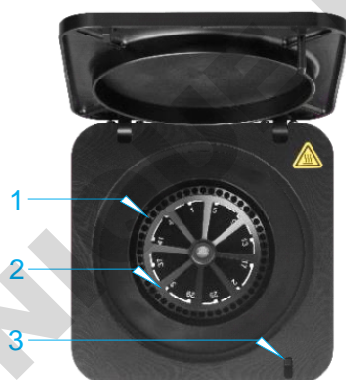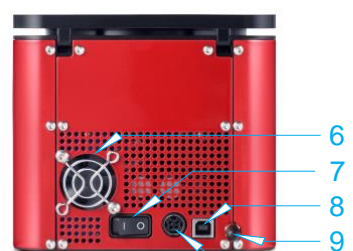
Consumables and Accessories

	Power adaptor **REF** MIC-PA	External power supply for the instrument. Provided with instrument.
	Tubes and caps **REF** MIC-TUBES	Strip of four reaction vessels with a volume range of 5 – 30 µL. Preloaded with silicone oil. Pre-packaged into a rack of 48 tubes, stacked together in a row of 5, and boxed as 4 x 5 stacks. Mic caps supplied loosely in a bag. One box provided with instrument.
	Tubes and racked caps **REF** MIC-TUBES+ RACKEDCAPS	Same as MIC-TUBES but the strip of four Mic caps are pre-packaged into a rack of 48 caps, stacked together in a row of 5, and boxed as 4 x 5 stacks. Can be used with MIC-RAPIDCAP.
	Capping tool **REF** MIC-CT	Allows for easy insertion of the caps into the tubes. Provided with instrument.
	Mic rapid cap tool **REF** MIC-RAPIDCAP	Allows for simultaneous capping of 48 Mic caps onto a rack of 48 Mic tubes.
	Mic SBS robotic loading block **REF** MIC-SBSLB	SBS dimension loading block to fit most liquid handling systems[6]. Loads 2 x 48 well Mic racks.

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[6] Talk to your liquid handling instrument manufacturer about incorporating the block into their software.

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
- Capping tool
- Mic tubes and caps (960 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® 8, 32-bit (English version) Operating System
- .NET Framework 4.5 of higher
- 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.

Running *micPCR software* on Macintosh operating systems

The *micPCR software* is currently not compatible with Macintosh systems. We recommend using a virtual machine program, ideally Parallels® or VMware® Fusion or Oracle® VM VirtualBox to run the *micPCR software* applications on a Mac without rebooting. This method will allow you to run macOS and Windows applications concurrently.

To dual-boot between macOS and windows, use Apple's boot camp (https://support.apple.com/en-au/HT201468). This method will only allow your computer to switch between booting up macOS or Windows partitions.

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.

| ⚡ mic M0000084 Idle | ⚙ mic M0000002 Idle | 📶 🖥 | Instruments connected to a PC via Bluetooth® and USB. |
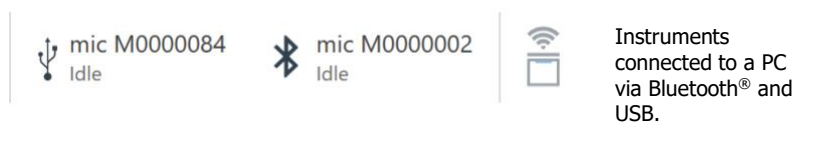
All connected instruments are now ready to be used.

Updating Software

Software and firmware updates are available for download at
www.biomolecularsystems.com/customer-resources/.
Please check the website periodically to see if new software and firmware updates are available.
Registered users will be notified via email upon release of a new software version.

Browse to the software heading of the Customer Resources page.
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 update setup file.
Release notes are also provided with each new build.

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

Upgrading Firmware

Some new releases of software will require a firmware update. The software will notify the user of the requirement to upgrade firmware upon selection of an instrument following the software update.

To achieve the firmware upgrade, the instrument must be connected to the PC via USB cable. Firmware upgrades cannot be achieved through Bluetooth®.

Select the *Upgrade Firmware* option in the drop-down list after selecting the *Instrument icon*.
The instrument will begin to flash the front LED indicator red to notify the user the firmware is being updated.

Upgrading to HRM

An 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.*

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.
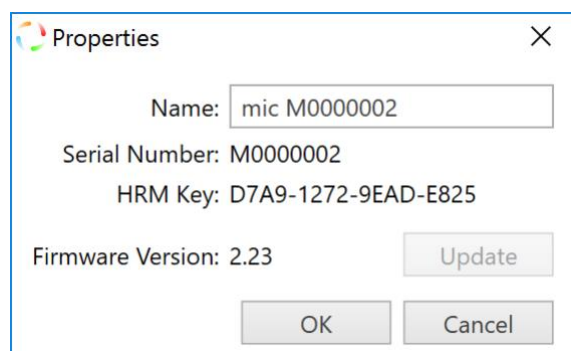
LED Indicator Colours

The LED indictor will change colour and flash during particular instrument operations.

Blue constant: The instrument is switched on and *Idle*.

Blue flashing: The instrument has been selected to *Start* a run. This instrument can no longer be selected by another user until the designated run has completed.

Green: The instrument is *Running*.

Green flashing: The instrument has completed the run successfully.

Red flashing: The run has been *Aborted*, the instrument has had an issue during the run, or the firmware is being upgrade.

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 5 - 30 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 rack to pipette reagents and samples into each tube.
The tubes are pre-packed into a loading rack, with the tube tab matching the tab on the loading rack. The loading rack is also compatible with a multichannel pipette 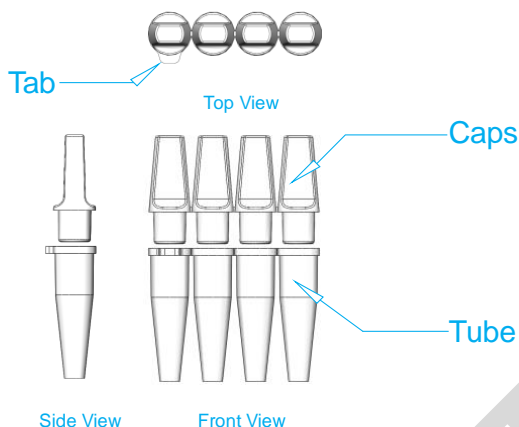(8-channel). For this strategy, the loading orientation switches to across the top of the loading block (A1, A2, A3…) instead of the standard down orientation (A1, B1, C1…). The software has provision to change the display of samples based on the type of layout selected (see Well Layout).

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.

Note: Ensure that only the first six tip positions are utilised and not the full eight.
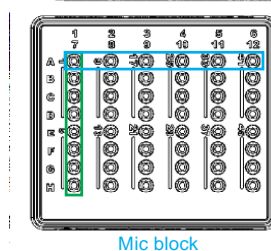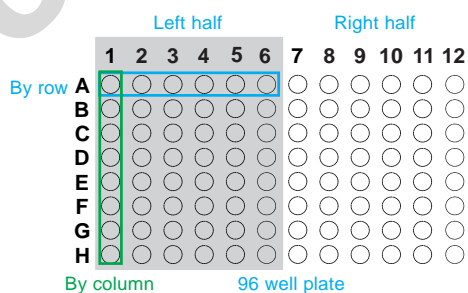
Wells highlighted in green follow the standard loading orientation (A1, B1, C1…) and wells highlighted in blue follow the multi-channel pipette loading orientation (A1, A2, A3…). The loading strategy will allow you to load half a 96- well plate per loading block.

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.

Thumb — Index finger

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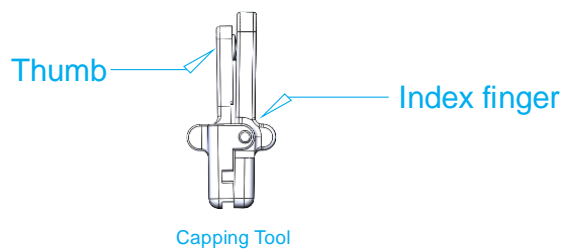
The tool is designed to clamp down on a strip of four caps. With your thumb and index finger on either side of the tool press the caps down into the strip of four tubes until firmly in place. Remove the tool by unclamping the caps.

Alternatively, if you are completing a full Mic qPCR run you can use the Mic Rapid Cap tool to quickly cap a full rack of Mic tubes.

Handle

Press Plate

Racks
Red Cap Rack (Top)
Black Tube Rack (Bottom)
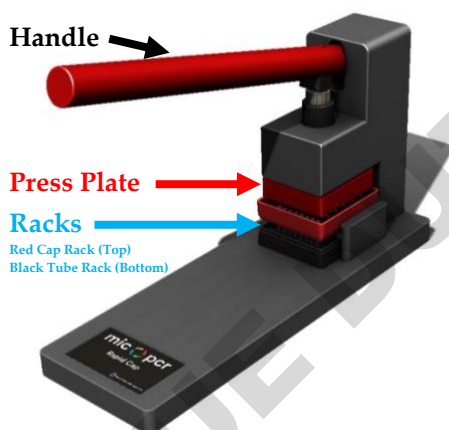
The tool is designed to clamp down a rack of 48 caps onto a rack of 48 Mic tubes. Flip over the cap rack and align the caps with the Mic tubes. Apply some pressure to loosely attach the caps to the tubes. Slide the racks into the Mic Rapid Cap tool with the red cap rack on top and aligned with the red press plate. Using the handle, press down the caps firmly into the tubes. Release the racks by raising the handle.

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.

The LED at the front of the instrument will flash green.

HOT SURFACE

⚠️

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 5 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
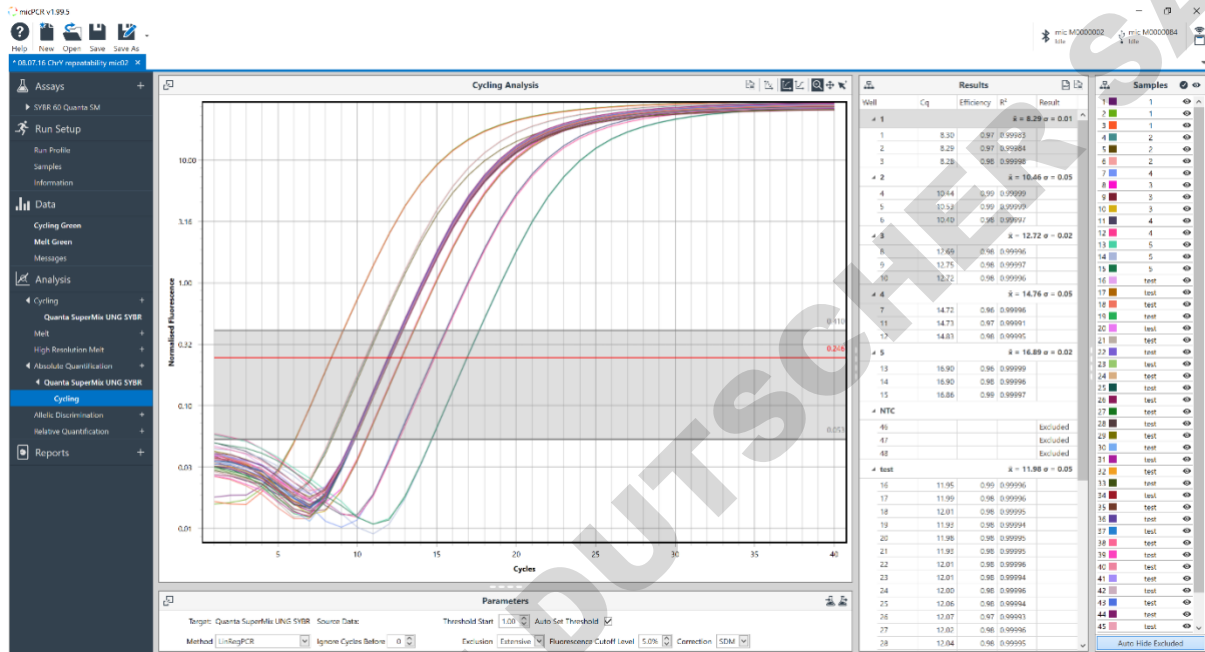
Tool Bar

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

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

New: create a new *Assay*, *Run*, *Run Template* or *Project* from a drop-down menu.

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

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

Save As: save an open *Assay*, *Run* or *Project* under another file name or as a *Run Template* or *Excel Workbook*.

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

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

Help Icon

The *Help* icon is used to access the following options:
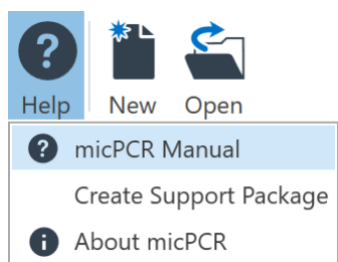
micPCR Manual: an electronic version of the user manual is stored within the software.

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 four file types:
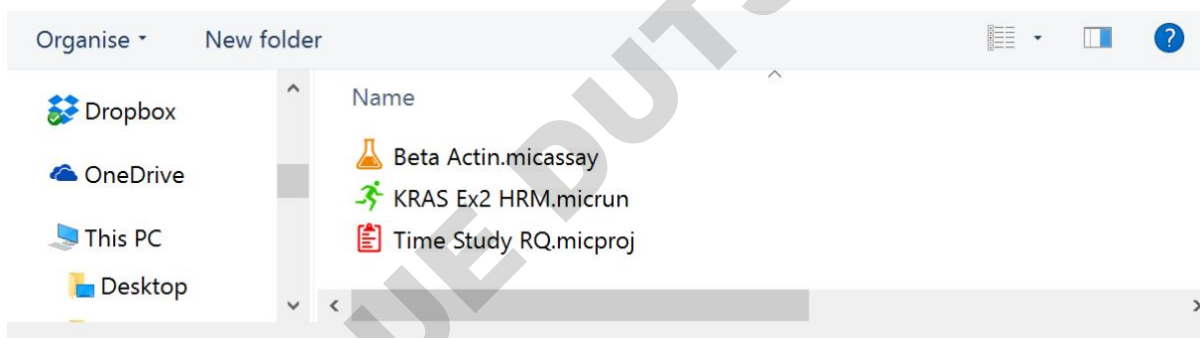
Assay File: contains all the information regarding a set of targets for a run (orange beaker).

Run File: contains the assays used, run profile, sample annotation, raw data and analysed data (green running man).

Projects: analysis of a combination of compatible runs (red clip board).

Run Template: contains a complete pre-run including sample annotation. A template can be used for repetitive runs using the same sample layout each time. Use *New* to open a run template (orange running man).

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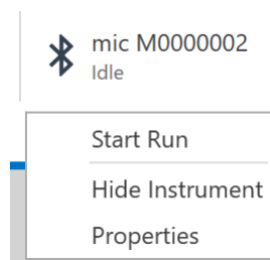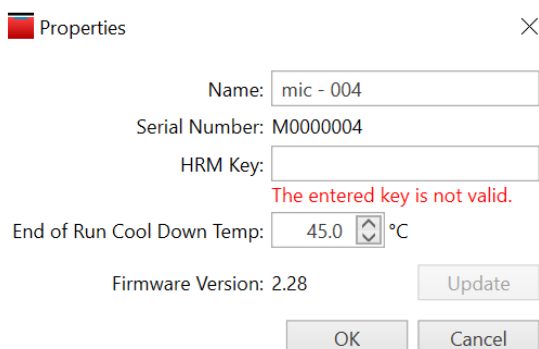
Start Run: runs begin by selecting the *Start Run* option. The *Start Run* option will only appear when a *New Run* has been initiated.

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.

> **End of Run Cool Down Temp**: The temperature that the instrument cools down to at the end of a run can be configured on the machine properties dialog. This temperature value is stored on the instrument. This temperature is best left at its default value of 45°C but can be increased in warmer operating environments if the mic consistently fails to cool down in time. Note that increasing this temperature will increase the temperature of the rotor at the end of a run.

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

Temperature Verification allows you to determine if the instrument is within specification using the Temperature Verification System (TVS). See Appendix A on how to operate the TVS. This option will only appear if the TVS is plugged in via USB.

Some options are only displayed when required:

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.

Instrument Communication Icon

The instrument communication icon is used to search for nearby instruments via both Bluetooth® and/or 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.

File Tabs
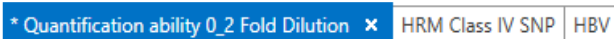
Every open file will be displayed with its name on a tab. Multiple files can be open at the one time. The file being displayed in the main window will be highlighted in blue. Files that need to be saved will have an asterisk just before the file name. If a file is linked to a run in-progress, selecting 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.
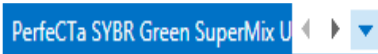
Navigator Bar
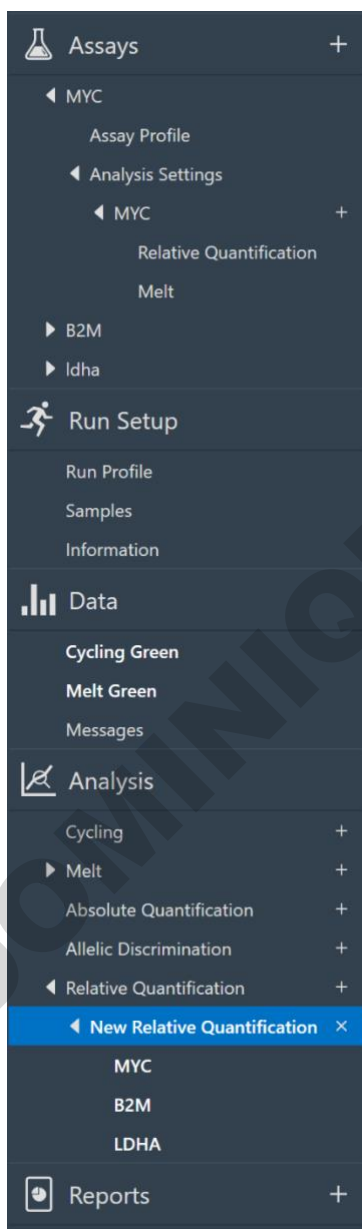
To the left-hand side of the main user interface is the *Navigator bar*. The Navigator bar allows you to view the different sections for an *Assay*, *Run*, or *Project*.

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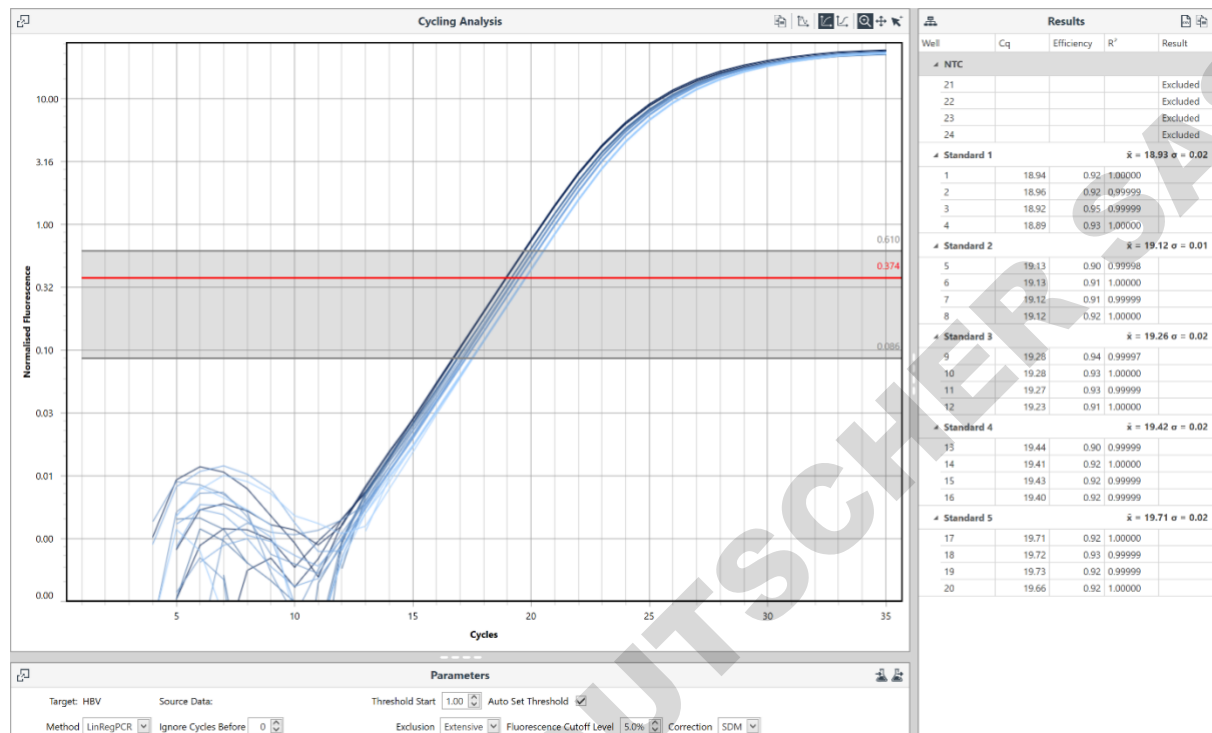
Raw *Data*: the data is updated in real-time during the run and is available after the run has completed. The graphs are scaled automatically during a run and can be scaled manually by expanding or contracting each axis.

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.

***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.

CSV Export or Copy to Clip Board

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

CSV: save the results as a CSV file.

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

Export Raw Data

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

Export Chart as Bitmap

Copy the graphs to a bitmap format for pasting into any third-party document processing software (e.g. Microsoft Word).

Scale the image to the size required.
To ensure you retain as much detail in the image as possible adjust the image to the largest view possible.

Select the *Copy chart to the clipboard* icon.

Paste the image into the third-party document processing software as a bitmap.
The image should be of sufficient quality for publication purposes.
For larger posters (e.g. A0) the images may appear slightly grainy.
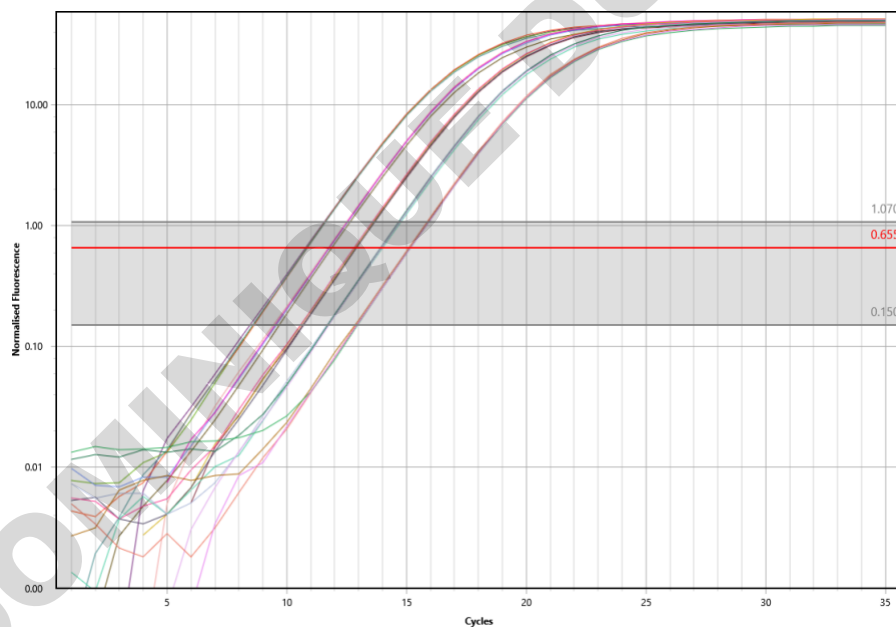
Result Table Organisation

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.
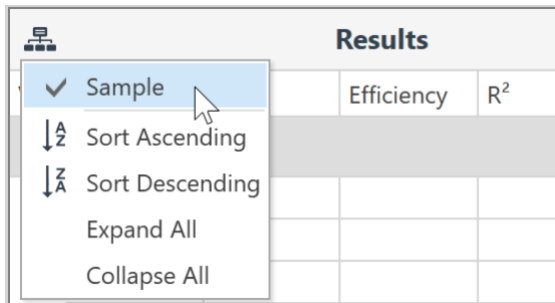
Sizing Bars

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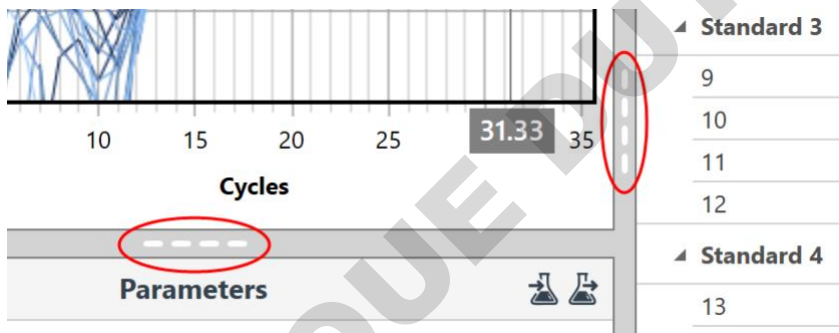
Graph Display Functions

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.
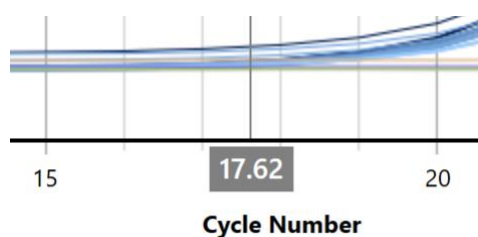
Parameter Settings

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. 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 *Select Named*, or select all samples by using the *Select All*, or deselect all of the samples by using the *Select None* options.
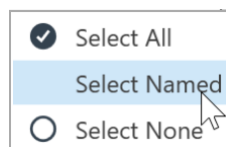
Hovering the mouse curser over a sample in a graph will highlight the sample by drawing the line thicker and displaying the name of the sample in a small text box along with the well-number. The sample bar in the *Samples selector* is also highlighted in black. Similarly, hovering over a sample in the Samples selector will highlight the sample in the graph.

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 *View* icon located on the sample selector. There is also an option to *Show All* or *Hide All*.

Samples Selector Grouping

Select the *Samples Selector Grouping* icon to view the sample selector based on *Source Run* (Projects), *Assays*, *Sample Groups*, *Sample Name* or *None* (well order).
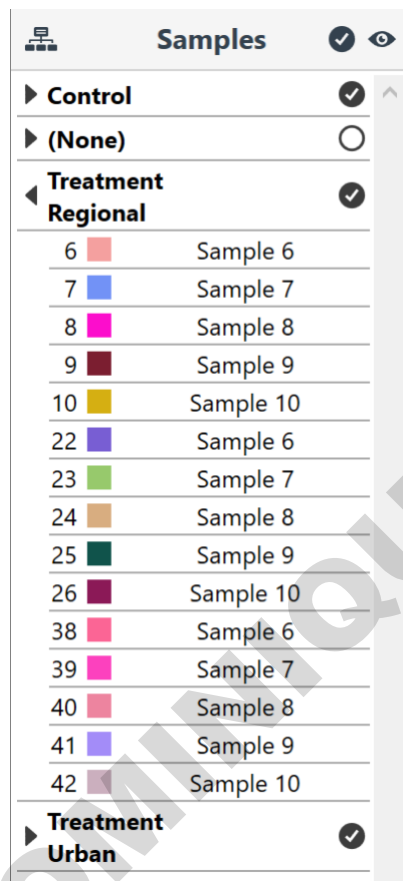
This option makes it easier to view or hide samples based on grouped types. You also have the option to *Collapse* or *Expand* the groupings to minimise cluttering on the sample selector.

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.
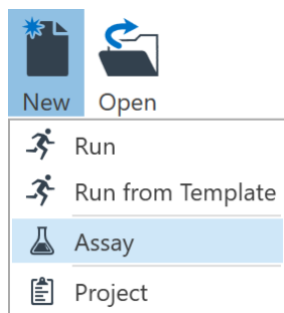
Assay Setup

Information

Select the *Chemistry Type*.
Choices include *Intercalating dye*, *Dual hybridisation Probes*, *Hydrolysis Probes*, *Molecular Beacon* probes, and *LUX® Primers*.
The type of chemistry selected will also set the default *Assay Profile*.
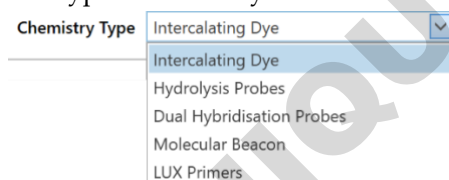
Enter the nomie of the amplicon target.
If using multiple targets for the one assay (multiplexing), select the *Add* button to setup another *Target*.
Multiple targets for the one assay are not permissible when using intercalating dyes.

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.

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

You must select a reporter dye so that the software can select the appropriate channel to acquire on. If your dye is not listed, then see the Dye Colour Chart located in Appendix C for further assistance.

For multiplex assays, add the additional *Targets* using the plus button. You need to ensure that each probe is labelled with a different channel dye. A warning will be displayed if two targets contain the same channel dye.

Targets

Name	❓	Reporter Dye	
Virus 1		🟩 FAM™	⌄ ✕
Virus 2		🟨 JOE™	⌄ ✕
Internal Control		🟧 ROX™	⌄ ✕

Optionally you can provide information regarding the quencher dyes used, but the information is not required by the software to execute the run successfully.

Oligo Nucleotides

Name	5' Label	Sequence	3' Label	Include	
Virus 1 Forward Primer		AGG CTG GTC AAC TCA GA		☑	
Virus 1 Reverse Primer		TTA CTG CAA GCT ACC GTC		☑	
Virus 1 Probe 1	🟩 FAM™ ⌄	ATT CCG TGT ACT AGG TT	⬛ BHQ® - 1 ⌄	☑	

Untick the oligonucleotide if you do not wish to use it in the reagent component list.

This might be the case if you have combined the oligonucleotides into one tube or you are using lyophilised material or a pre-made master mix (e.g. kit).

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.

Oligo Nucleotides

Name	5' Label	
Factor V Forward Primer		
Factor V Reverse Primer		
Factor V Probe 1	🟩 FAM™	⌄
Factor V Probe 2	🟨 JOE™	⌄

Contains Alleles ☑

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.

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.

Reaction Setup

Complete the *Reaction Setup* table (Myra option).

Enter the name of any component required for the reaction.
Ensure you enter the required volume for each component. A warning will be displayed if the total volume of the components exceeds the total reaction volume.
Oligonucleotides are automatically populated into the table unless unticked in the oligonucleotide table. A default name using the Target name will be displayed. Any edited name change will be recreated in the table.

Select the type of tube used to store the component (e.g. 1.5 mL tapered flip cap tube).
This will assist the Myra software during deck layout.

Enter the volume required for each component.
The default sample volume is 1 μL. For all Mic runs a template must be used.
The default total volume is 25 μL. Please change to the volume required. This volume will be populated in the Profile Editor.
If using a premade master mix (e.g. kit) then ensure that the volume used is such that the water volume is zero. The Myra will aliquot the pre-made master mix directly into the reaction tubes without the need to create an intermediate mix.

Reaction Setup

Components	Tube Type	Volume (μL)	
Water	Generic 5ml Screw Cap Tube	8.75	
Source Template	Unassigned	1	
Factor V Forward Primer	Generic 1.5ml Flip Cap Tube	2	
Factor V Reverse Primer	Generic 1.5ml Flip Cap Tube	2	
Factor V WT	Generic 1.5ml Flip Cap Tube	2	
Factor V MUT	Generic 1.5ml Flip Cap Tube	2	
10X PCR Buffer	Generic 1.5ml Screw Cap Tube	2.5	×
25 mM MgCl2	Generic 1.5ml Screw Cap Tube	2.5	×
2.5 mM dNTP	Generic 2ml Screw Cap Tube	2	×
5 U/uL Taq polymerase	Generic 500μl Screw Cap Tube	0.25	×
Type here to add a new reagent	Unassigned		

Total Volume (μL)   25

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.
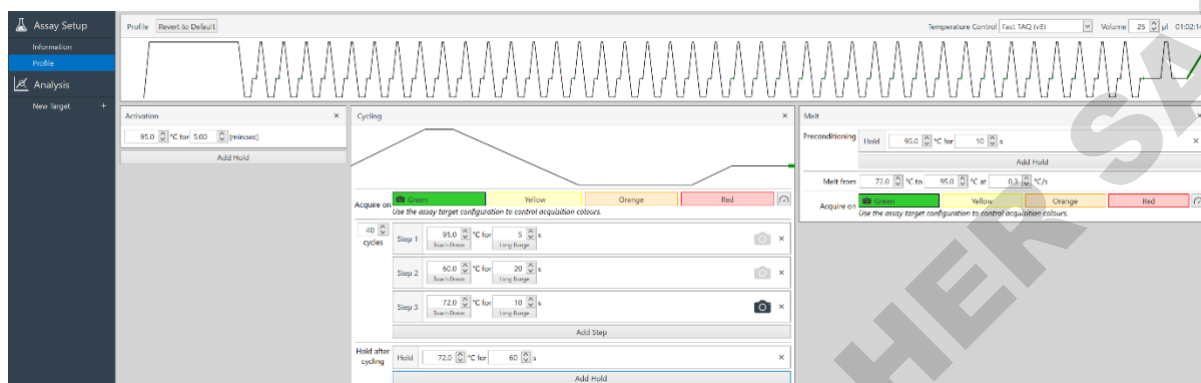
Initial Holds

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. The minimum temperature that can be set during an initial hold is 35°C. It is not recommended that temperatures below 40°C be set after holding at a temperature > 80°C.

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.

Pre-Cycling

Add an initial cycling that does not acquire. This will allow for applications where the initial stages of the PCR effect the behaviour of the signal. For example, when applying touchdown, where fluorescence is inversely proportional to temperature thereby resulting in a linear increase in signal. Although, the Ignore cycles option can be used during analysis for some users it is beneficial not to see the change in signal in the raw data.

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 level the run should start with.
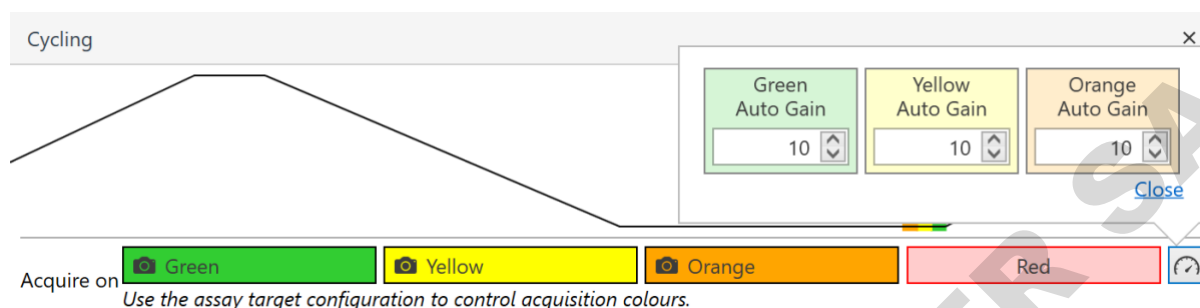
When using chemistries that start with a high background and reduce in signal during cycling, such as quenched FRET dual hybridisation probes or Plexor®, set the baseline to a high level. The default option for such chemistries is 70 units. The instrument will adjust the gain of the selected optical 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 a 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). The tube with the highest fluorescence is used to achieve optimisation. Specific tubes can be selected to run the gain optimisation on (see Run Profile in Run Setup section).

ATTENTION

Ensure that tubes from a previous run are not left over into the new run. These tubes may have significantly higher fluorescence values compared with the new reactions. This will result in the old tubes being used to optimise gain resulting in lower fluorescence values for a new reaction tubes.

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 99ᵒC.

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

Touchdown: can be enabled to decrease or increase the temperature during the initial cycles.
Set the decrement in temperature required using a positive number and increment using a negative number.
Enter the number of cycles per decrement.
Indicate the number of times you wish to apply the decrement.
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. For a standard touchdown, select 1ᵒC decrement at 1 cycle each for 10 cycles. This will decrease the temperature 1ᵒC each cycle for the first 10 cycles. Alternatively, you could set a one-off increase in temperature of 10ᵒC at cycle 20.

Long Range: can be enabled to increment the hold time of the selected step.
Set the number of cycles, the increment in time per cycle and the number of instances required.
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 than 1°C/s will generate data that may not be substantial enough to provide meaningful information.

Melt						✕
Preconditioning	Hold	95.0 ◊ °C for	15 ◊ s			✕
	Hold	45.0 ◊ °C for	60 ◊ s			✕
			Add Hold			
Melt from	50.0 ◊ °C to	80.0 ◊ °C at	0.3 ◊ °C/s			
Acquire on	📷 Green	Yellow	Orange	Red		⌒

Use the assay target configuration to control acquisition colours.

Adjust the gain settings for the melt acquisition.
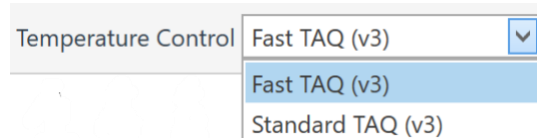
Only channels selected in the Information section will be available in the melt.

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.

Temperature Control | Fast TAQ (v3)
Fast TAQ (v3)
Standard TAQ (v3)

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).
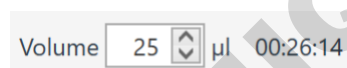
Due to the fast temperature ramping speeds, possible on the Mic instrument, failure to apply a slow rate of heating for standard *Taq* polymerases, without using longer hold times at anneal (> 20 s) or three step cycling, may result in poor amplification, with reduced analytical sensitivity and amplification 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 page 21 regarding the use of *water tubes* as another measure to ensure the highest level of temperature uniformity.

Volume | 25 | µl | 00:26:14

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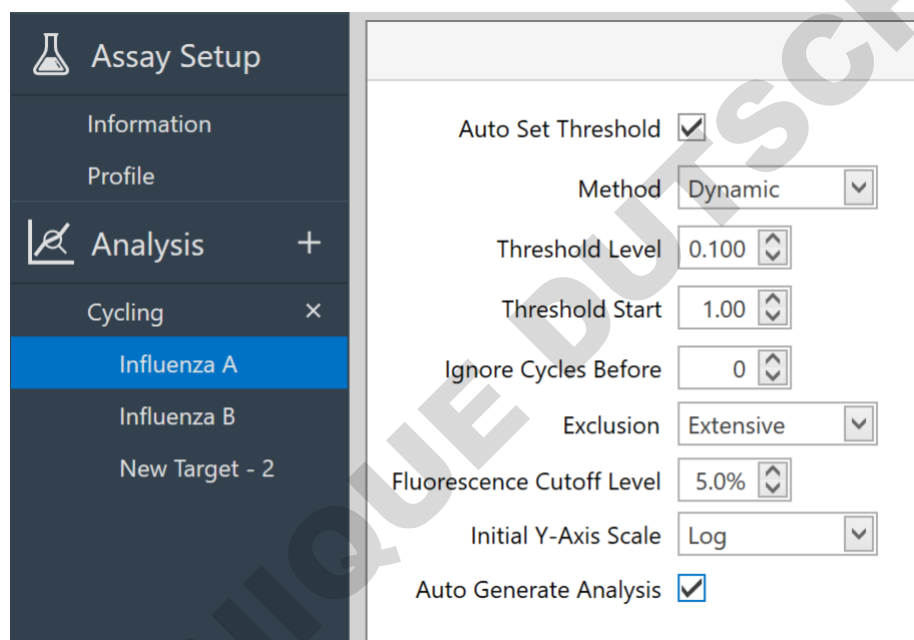
This feature enables you to store all analysis parameters within the assay, thereby avoiding continually having to edit parameters for each new analysis.

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. The default method selected will depend on the Chemistry type selected for the assay (e.g. Hydrolysis probes will default to Dynamic method).
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.

Select an Auto Exclude function.

Select what level of exclusion to apply for cycling analysis.

Chose the Initial y-axis scale.

The default is log scale, with the option to display the normalised curve in linear scale.

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 Filtering level required.
Options range from none too high. The default is medium.

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.
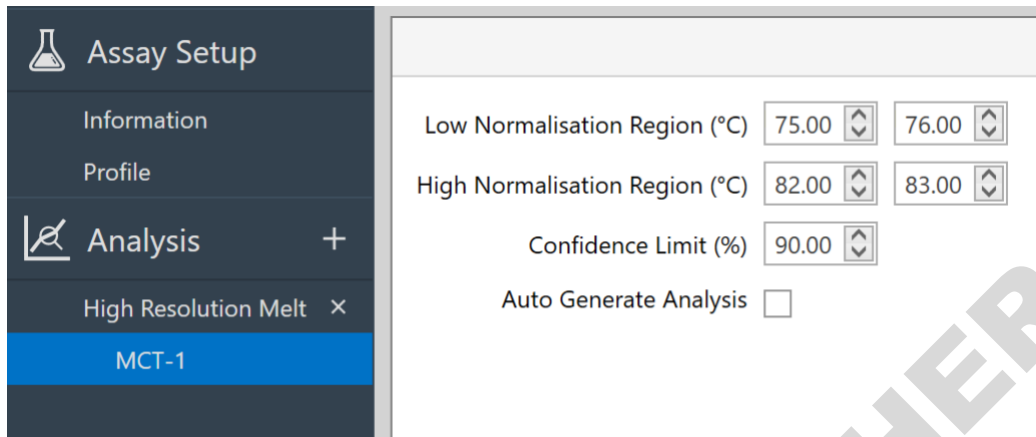
HRM

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.
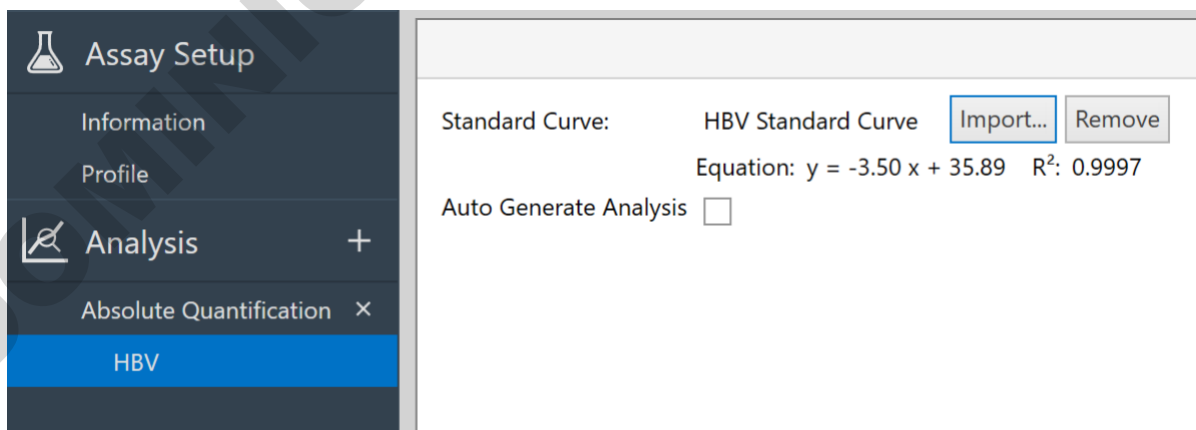
Enter a confidence percentage threshold.
Samples below this value will not be reported with a genotype but rather will be reported as unknowns.

Absolute Quantification

Import a standard curve.
Select the required standard curve file (*.micsc) from the file location it was previously exported to.

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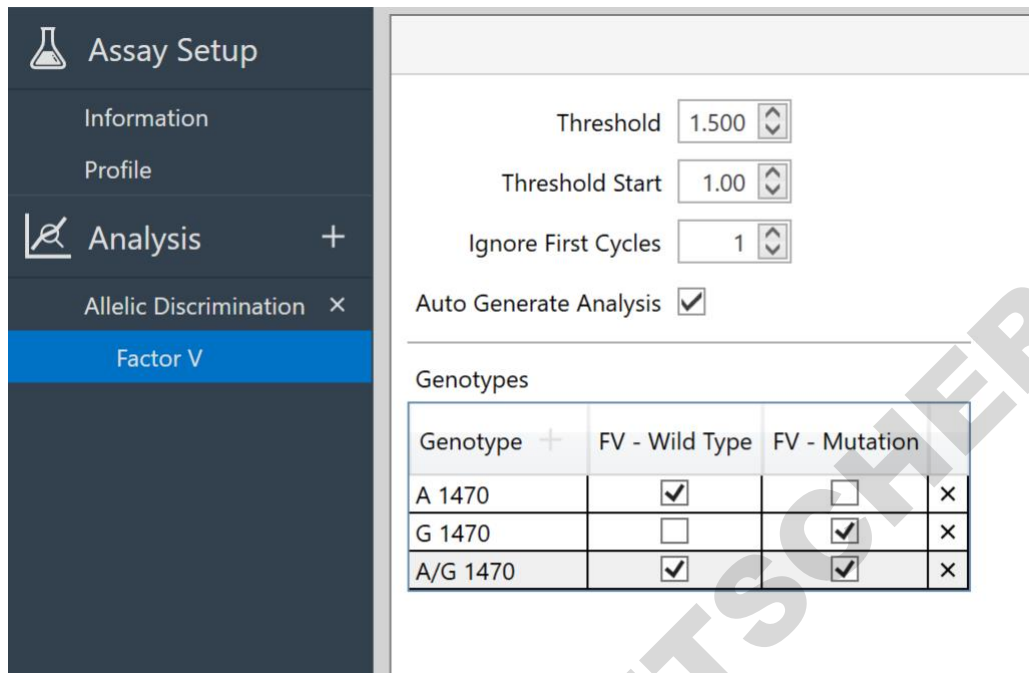
Allelic discrimination uses real-time kinetic data from two or more channels to genotype samples.

Enter a Threshold level that allows sufficient differentiation of each allele on the normalised real-time curve.

For some assays, low level amplification signals might be detected for a probe in the other channels. This may occur due to cross reactivity between probes for the target, cross talk between dyes or self-hydrolysis of the probes.

Change the Start Threshold or Ignore the first number of cycles used for normalisation.

Enter the Genotype names required.

Link each genotype to one of the alleles (probes).

For heterozygotes select both alleles (probes). Up to four probes can be used per target.

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 1 (behaviour is similar to ΔΔCt method and not recommended).
Import a standard curve to run the efficiency calculation with.

Select a colour to represent the gene in the analysis chart.

NOTE Ensure that the correct Cycling analysis parameters are set for each target.

Identifier

The *Identifier* software uses a set of rules to auto call presence or absence of a target(s) in an unknown sample. Controls are also utilised to make appropriate decisions regarding the result of each sample and the run as a whole. These rules have to been defined for each test. See Identifier in the Analysis section to learn more about how to setup the logic engine.

Evaluate your rules by using *Test Cases*.

You can test the rule you have created; or you can create a typical set of conditions first for all of the scenarios you want to catch and then create and tweak your rules until the results match what you have defined in your tests.

Add a test case by selecting the + icon at the top.

Provide a simple *Description* of the case, and the *Expected Decision*.

Below the test case, enter the values for a *Target*(s) to evaluate the test case. For example, enter the C_q value for the target of interest to ensure you can observe a positive call in the *Actual Decision*. Ensure that you nominate the correct *Sample Type*. A blank value indicates no calculable C_q or efficiency (i.e. a match for the "Has No Value" test).

Create multiple test cases to evaluate each decision using various conditions for each rule set.

The number of test cases that *Pass* or *Fail* are reported at the top along with a tick or cross next to the test case.

Test Cases			5 Pass, 0 Fail +

	Description	Expected Decision	Actual Decision	
✓	Positive HPV	Positive for HPV	Positive for HPV	×
✓	Negative HPV	Negative for HPV	Negative for HPV	×
✓	Fail run	WARNING: Run F...	WARNING: Run F...	×
✓	Fail sample	WARNING: Samp...	WARNING: Samp...	×
✓	Contamination	WARNING: Cont...	WARNING: Cont...	×

Test case: Positive HPV   Expected decision: Positive for HPV

Target	Sample Type	Quantity	Value	
HPV	Unknown	Cq	19	×
IAC	Unknown	Cq	23	×
				×

Auto Generate Analysis

By selecting this option, the software will generate the analysis, using the predefined parameters, once the run completes. The option is available for all analysis types, except Relative Quantification, which requires multiple assays grouping to complete analysis.

Saving a New Assay

Once all the parameters have been entered for the assay, the assay should be saved.

Assays can 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.

Alternatively you can save the assays into another personal or shared directory including network drives or external storage drives such as a USB Flash drive.

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.
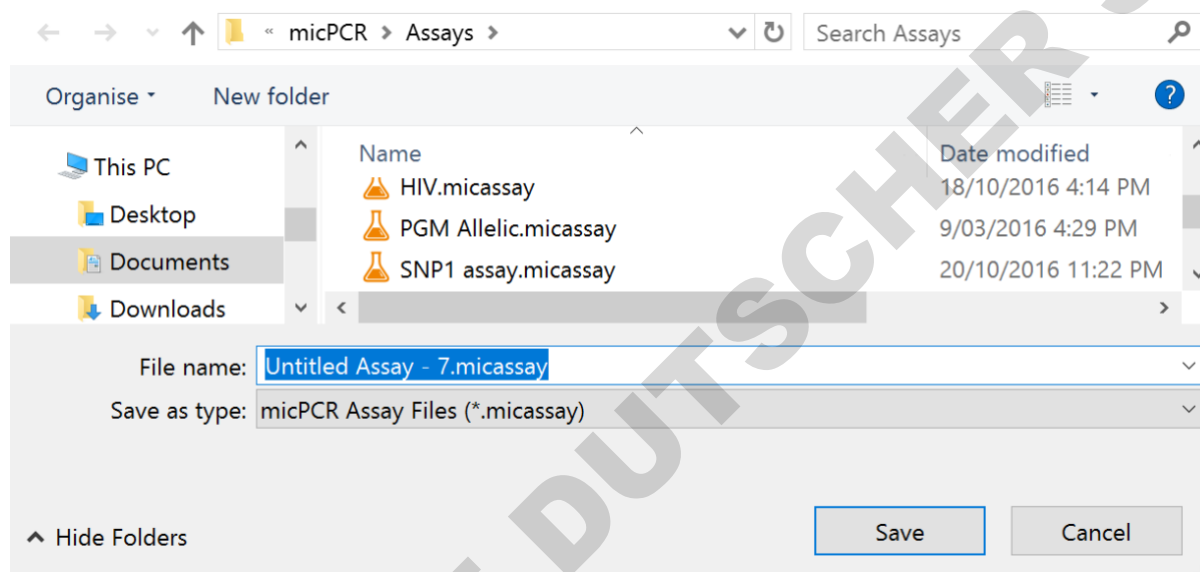
Creating a New Run

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 and Allelic Discrimination analysis will not work without pre-defined assays.
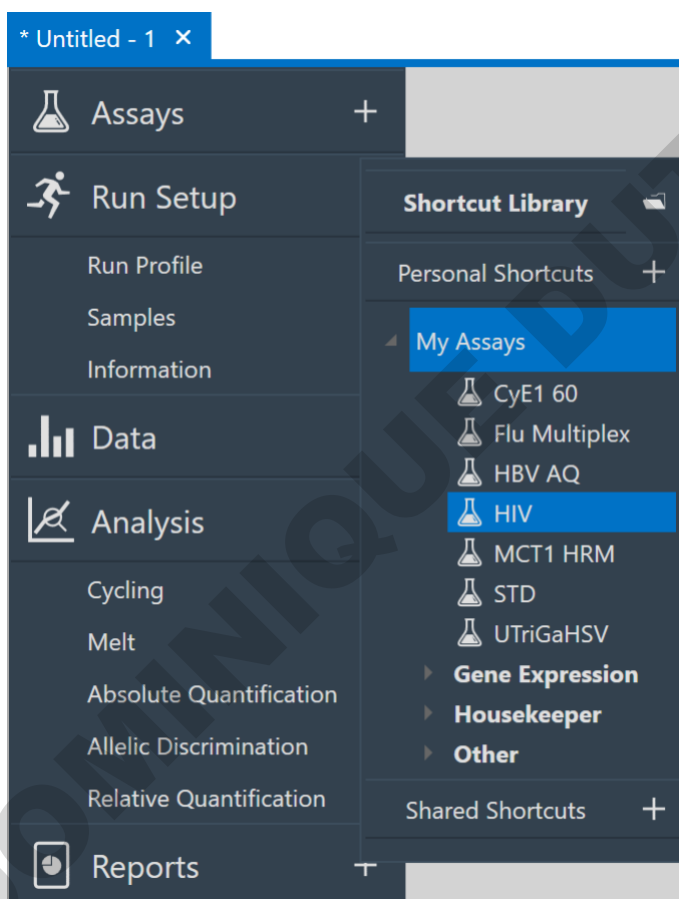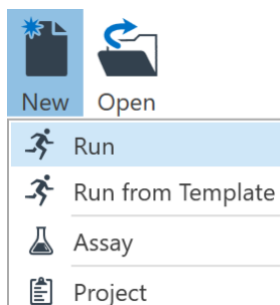
Adding Assays

Select the *Assay*s required for the run by selecting the *Add* button.

You can select from any library of assays displayed next to the navigator bar.
By default, the micPCR Library will be displayed.

Select an assay from any directory using the file icon.

Use the file explorer to locate the assay you require from any location including network drives or external hard drives such as a USB Flash drive.
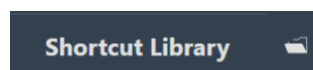
You can create a new assay library *Shortcut*.

Select the + button next to *Personal Shortcuts* then browse and select the location of the new directory you wish to use as a personal assay library.
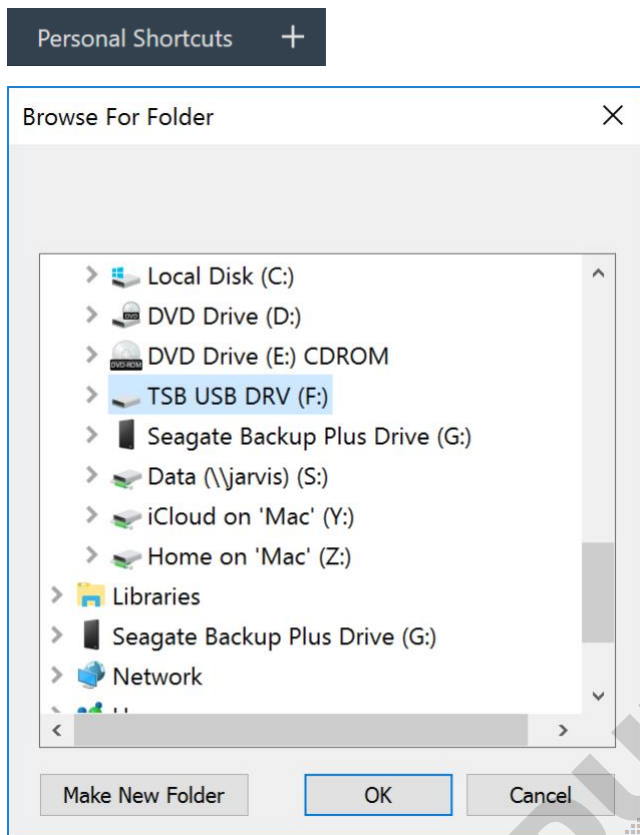
Create a *Shared* assay library shortcut.

If you have multiple users sharing a PC or network drive you have the option to create a shared location for all of your assays.
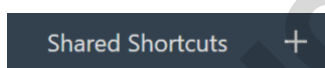
Select an assay from any directory using the Browse icon.

Use the file explorer to locate the assay you require from any location including network drives or external hard drives such as a USB Flash drive.

Alternatively, you can create a new assay from the run file.

Enter the assay details as described in section Create Assay, and then save the new assay file by entering a name in the field provided in the Navigator bar and selecting the save icon.
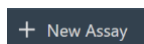
You can Edit or Delete assay libraries from the list.
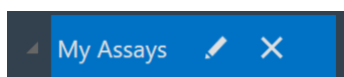
The *Information*, *Assay Profile* and *Analysis Settings* can be viewed and edited once the assay is selected. Remove assays by using the delete button next to the assay name.

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.
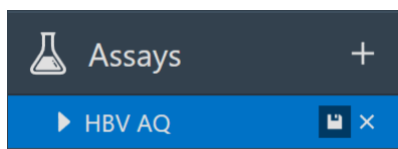
Run Setup

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 *Standard 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.
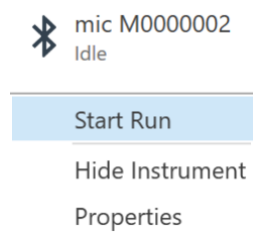
Once the instrument is chosen, begin the run by selecting the *Start* option from the drop-down list.

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.

At this point the instrument LED indicator will be flashing blue indicating to other users the instrument has been 'booked' to start a run. No other user can start a run on the instrument until it has completed this run. On another user's PC, the instrument will be displayed as *Busy*, when they go to select it.
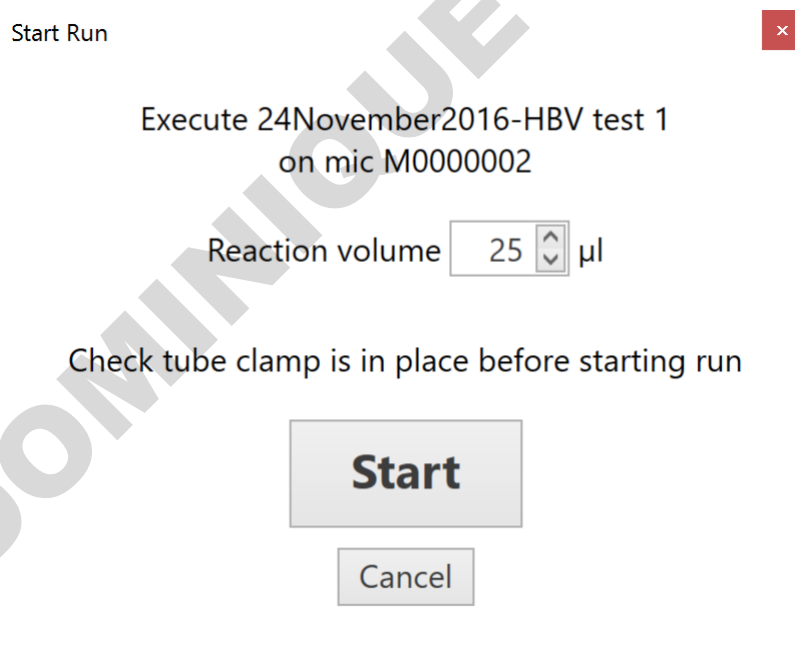
To execute the run, click the *Start* button in the Start Run dialogue box.

The instrument will automatically lock the lid, centrifuge the samples down, and then the run profile will begin. The LED indicator will turn green to notify a user the instrument is running.

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. A default table is provided but the position of the columns can be changed by dragging a particular column header left or right.

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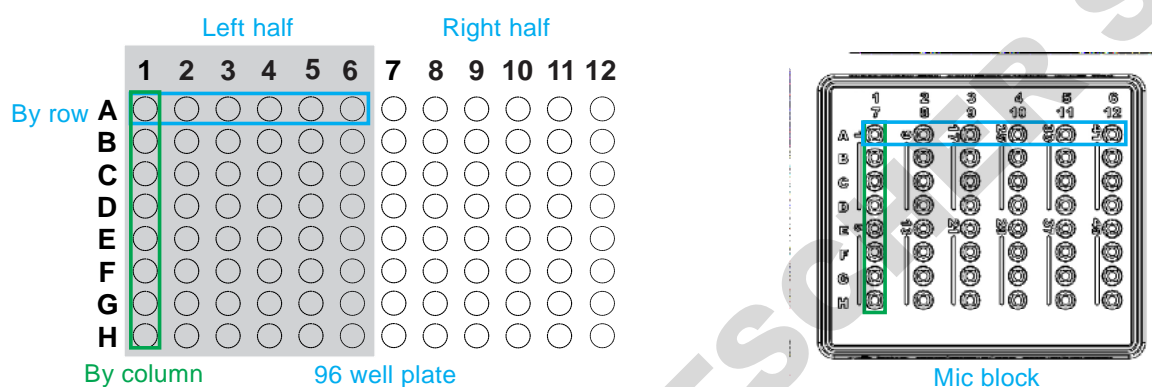
Display wells as either numerical (1, 2, 3…) or alpha numeric (A1, B1, C1).

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 (Not available yet).

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 (Not available yet).

Sample Concentrations

Select the units to report.

There is a list of units to choose from. Alternatively, enter your own units, in the provided text box.

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 = 1×10^3 = 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).	Concentration Units Copies/µL ▾ 20000
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).	Concentration Units Copies/µL ▾ 20000 10000
Now highlight all the cells required to complete the dilution series and replicates.	Concentration Units Copies/µL ▾ 20000 10000

Click on the Auto fill icon.

Concentration	
Units	Copies/µL
	20000
	20000
	20000
	10000
	10000
	10000
	5000
	5000
	5000
	2500
	2500
	2500
	1250
	1250
	1250

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

Multiplex Standards

You can enter standard concentrations for each channel individually.

Select the *Toggle between single and multiplex standards* icon to use the option of entering multiplexed standards. You must ensure that the assay has been setup as a multiplex.
Switching between single and multiplex is possible, however, only one type can be viewed and applied during analysis.

Green Channel Standard...	Yellow Channel Standard...	Orange Channel Standar...	Red Channel Standards...
%	%	%	%
9	35	1	55
1	55	9	35
25	25	25	25
55	9	35	1

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.

Available Assays
MYC
B2M
ldha

Optional Columns

Additional columns can be added to the *Samples editor* using the *Select visible sample data columns* icon. The following columns can be added or removed from the table:

Standard Concentration: concentrations are used for Standard curve analysis.

Input DNA concentration: note the starting amount of DNA per reaction.

Input RNA concentration: note the starting amount of RNA per reaction (RT-qPCR)

RIN: provide an RNA integrity number for each input RNA.

Comment: provide additional information about the samples.

Groups: allocate groups for analyses such as Relative Quantification.

- ✔ Standards Concentration
- Input DNA Concentration
- Input RNA Concentration
- RIN
- Comment
- ✔ Groups
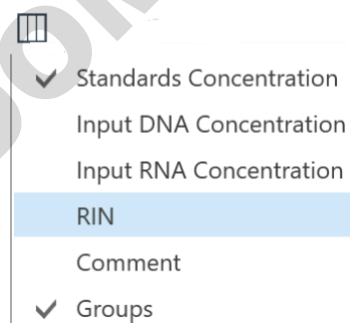
Import Samples

Import sample information from a number of file types to simply sample annotation.

	A	B	C	D
1	Well	Sample ID	Concentration	
2	1	Sample 1	82	
3	2	Sample 2	158	
4	3	Sample 3	569	
5	4	Sample 4	924	
6	5	Sample 5	717	
7	6	Sample 6	731	
8	7	Sample 7	199	
9	8	Sample 8	110	
10	9	Sample 9	184	
11	10	Sample 10	812	
12	11	Sample 11	113	
13	12	Sample 12	246	
14	13	Sample 13	711	

Select the Import Samples icon to import sample information from another source.

You can import from any comma delimitated, tab delimited or space delimited files.

Browse and select the file to import.

Select the fields to import and into which column of the Sample Editor.

Once the run file is selected a table will display all the fields in the file. You have the option to select the type of delimitation (e.g. comma) and how many rows should be ignored before capturing the data.

Next, chose the fields to import by linking the column from the files to one of the columns in the sample's editor using the drop-down menu.
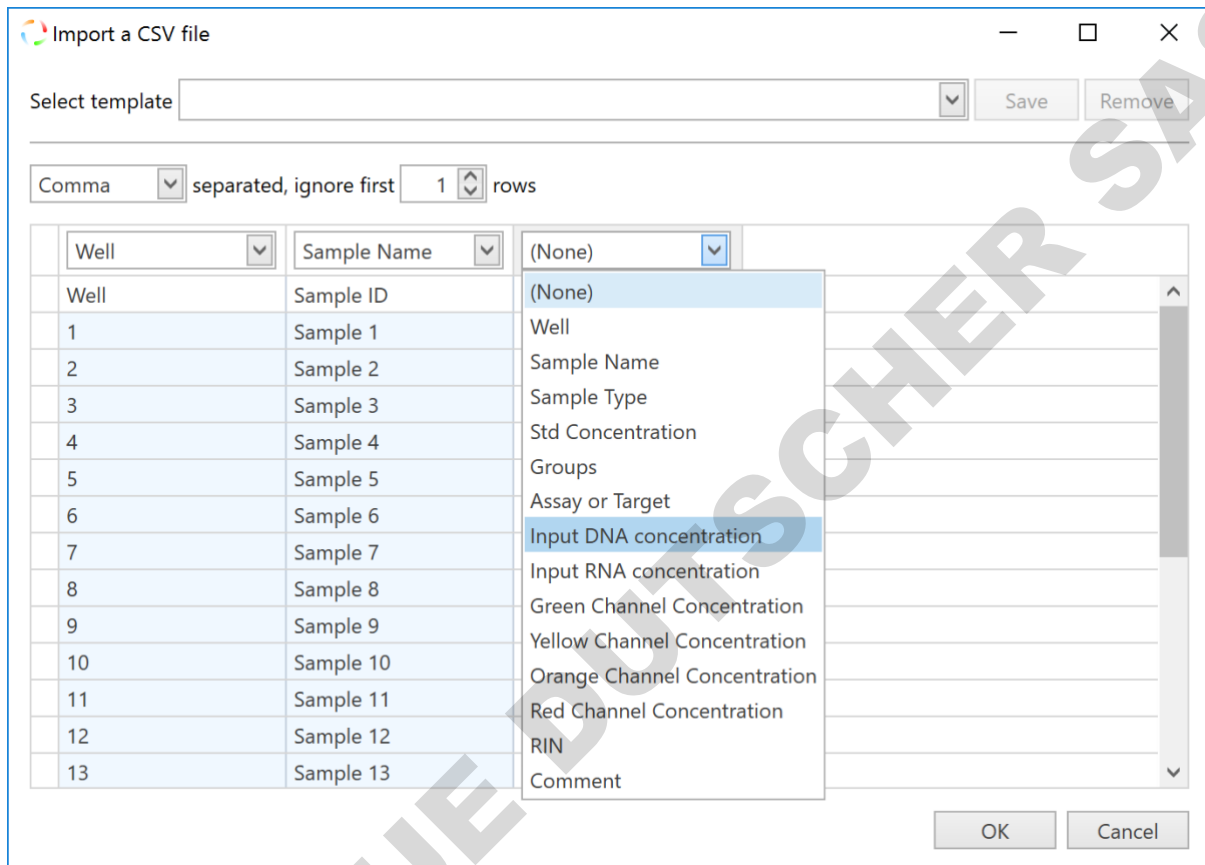
You have the option to save the import style as a template.

This will allow you to complete the import faster without having to re-do the matching when conducting repetitive runs.

Sample Editor Warnings

Various warnings will be displayed if annotations have not been completed correctly. Some examples include:

- If standards have been selected as *Type* but no values have been entered into the *Standards Concentration* field.
- Assays have not been linked to an edited Sample row.

⚠ The standard in well 1 does not have a concentration defined.
Some named samples do not have an assay assigned to them.

Lock Down Samples Editor

All of the fields can be locked down to prevent accidental changes occurring. Once locked the none of the fields are annotatable until unlocked again.

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).

The Integrity of the data is also reported, as part of the 21CFR11 recommendations (see Appendix B).

Creating Templates

Templates allow the user to set-up runs that will be used repetitively. For example, you may want to run a group of assays using a particular set of controls and standards in specifically allocated wells all the time.

Create a Template

Start a *New Run*.
Complete your run using the same methods described above.

Select the down arrow next to the *Save As* button, then select *Template*.
Save the template into the *Template* library located in Documents/Bio Molecular Systems/micPCR/Templates.
You also have the option of creating subfolders within the library.

Opening a Template

Select *Run from Template* option in the *New* button on the tool bar.
The run will open with all the saved parameters including sample annotation.
Start the run as described above (see Starting a Run).
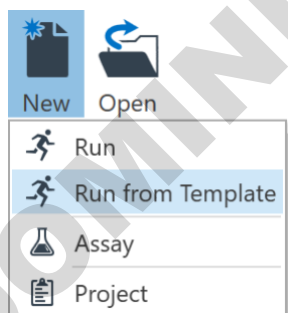
During a Run

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.

Modifying a Profile during a Run (Optional)

The *Run Summary* banner also has three functions to control the run: *Skip*, *Add 5 Cycles* more to the run, and *Abort* a run.

Skip a Section of the Profile

Use the skip function if you wish to move to the next section of the profile.
You may choose to skip the last number of cycles of a run profile if you believe there has been a sufficient number of cycles to generate amplicon and would like to commence the melt.

Add another 5 cycles to the run

Use this function if you believe you required more cycles to thoroughly complete amplification.
You can only add another five cycles while the cycling is still running, up to a maximum of 60 cycles.

Aborting a Run

You can stop the run at any point by selecting the Abort function in the summary window.
The instrument will cool to a safe temperature before disengaging the lid lock.

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.

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.
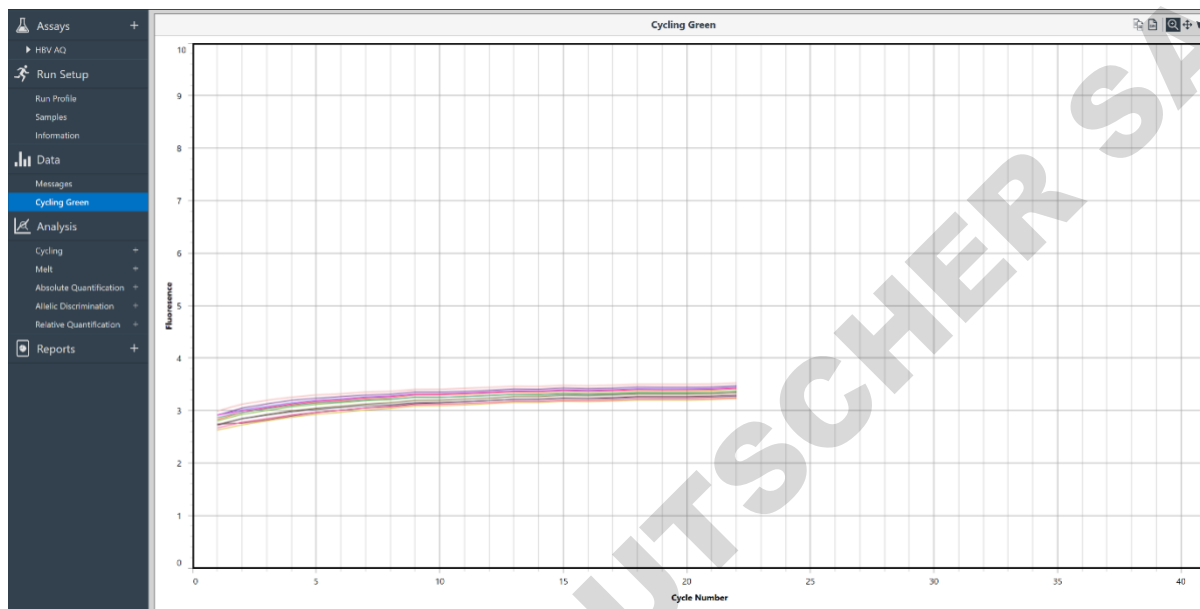
Melt data is plotted as temperature (*x*-axis) against fluorescence (*y*-axis) with a maximum fluorescence value of 100 units. Again, the graphs are auto-scaled depending on the *Adjust Gain Settings* option chosen.

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

During analysis, all changes to parameters will be logged in the messages list once the run file is saved. This procedure allows the software to conform to 21CFR11, by creating audit trails (see Appendix B).

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*. 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 type 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 (optional upgrade version)
5. Allelic Discrimination
6. Relative Quantification
7. Identifier
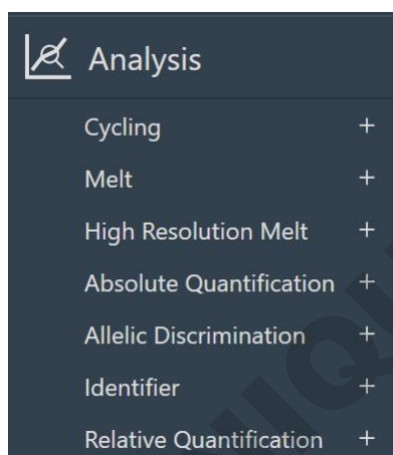
Then select the Target to analyse from the list of options.
The targets listed will be based on the assays chosen before starting the run. For multiplex assays each target will be shown as part of the assay. For allelic discrimination assays the added probe names will also be shown along with the target name.

The target name selected will be displayed in the navigator bar below the analysis type. You may edit the name by double-clicking on it.
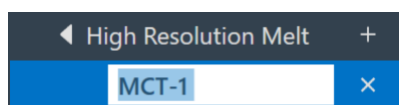
Multiple analyses are possible for each run, with the analysis being viewed highlighted blue on the navigator bar.

Delete any analysis by selecting the *Delete* button next to the target 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 target that was chosen.
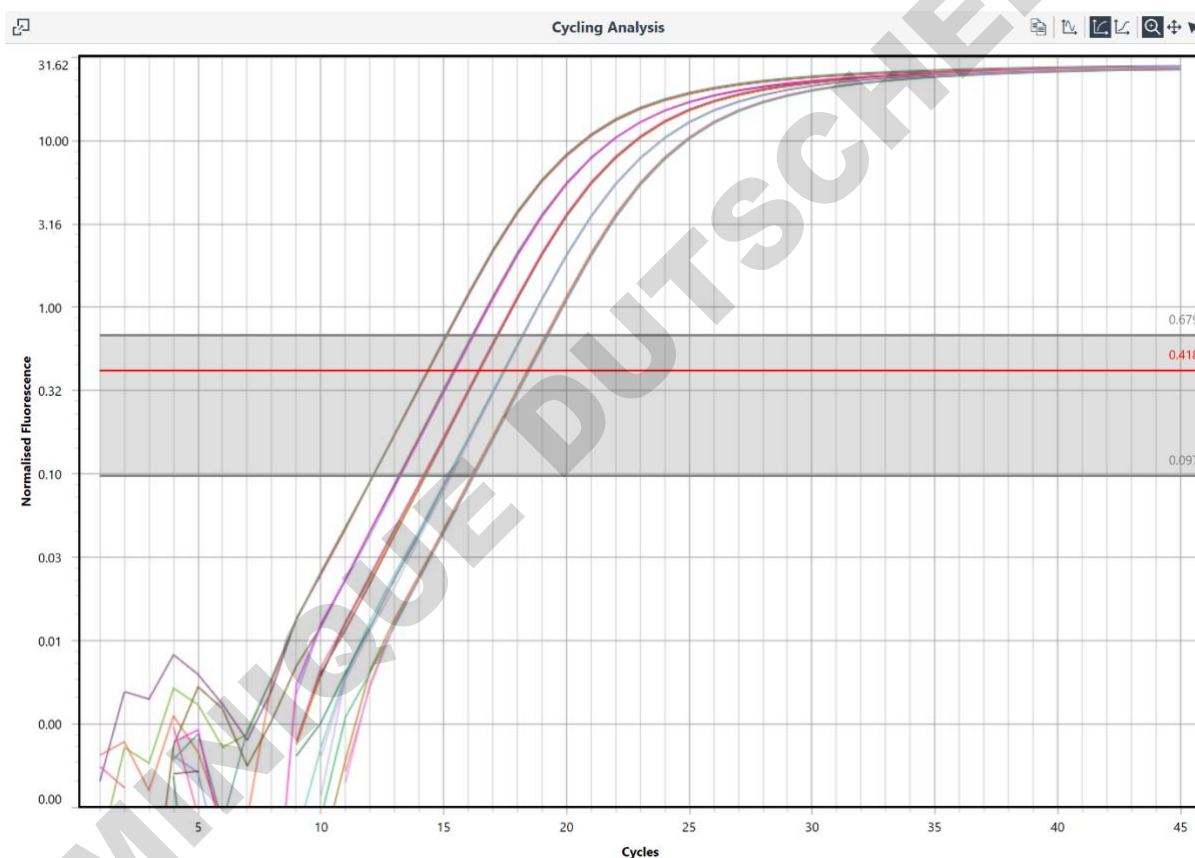
Graph Types

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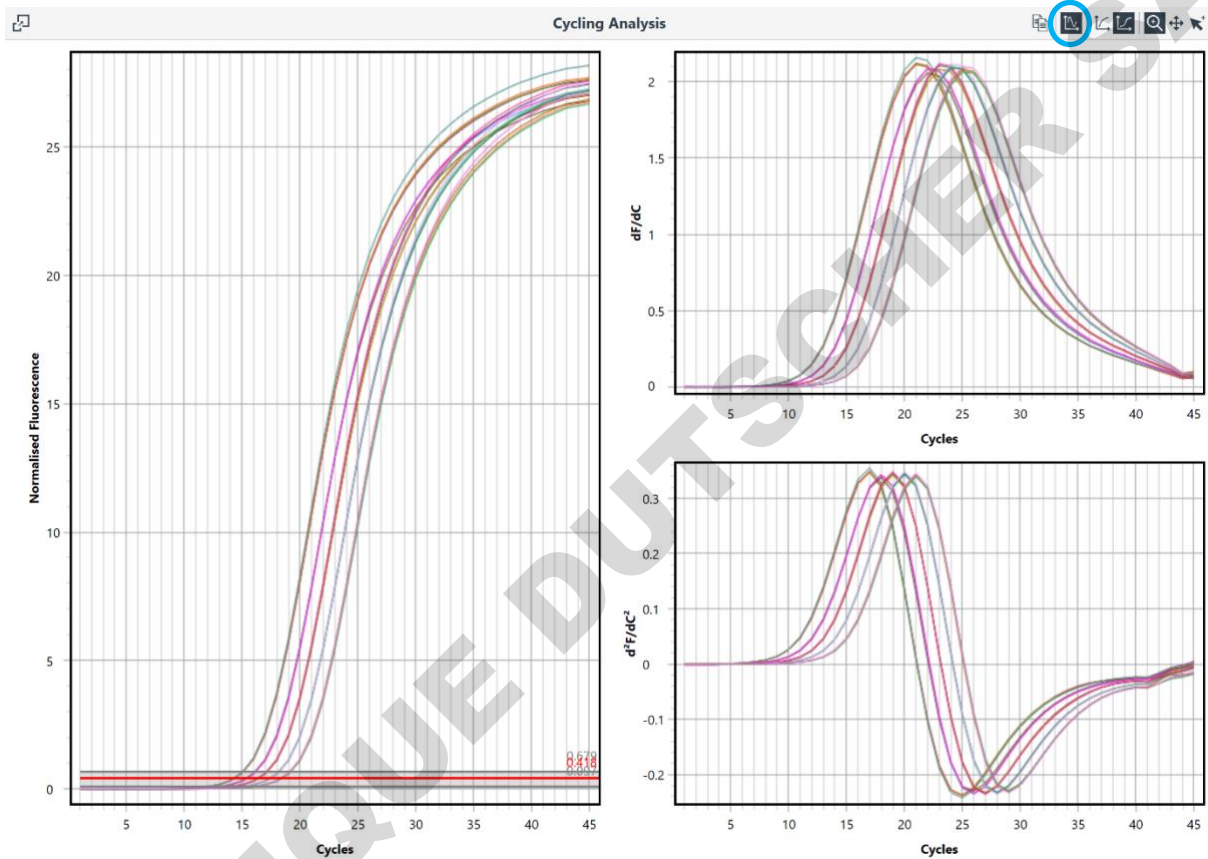
Linear *y*-axis: display the baseline corrected cycling data with the *y*-axis in linear scale by selecting the *Linear y-axis* icon.
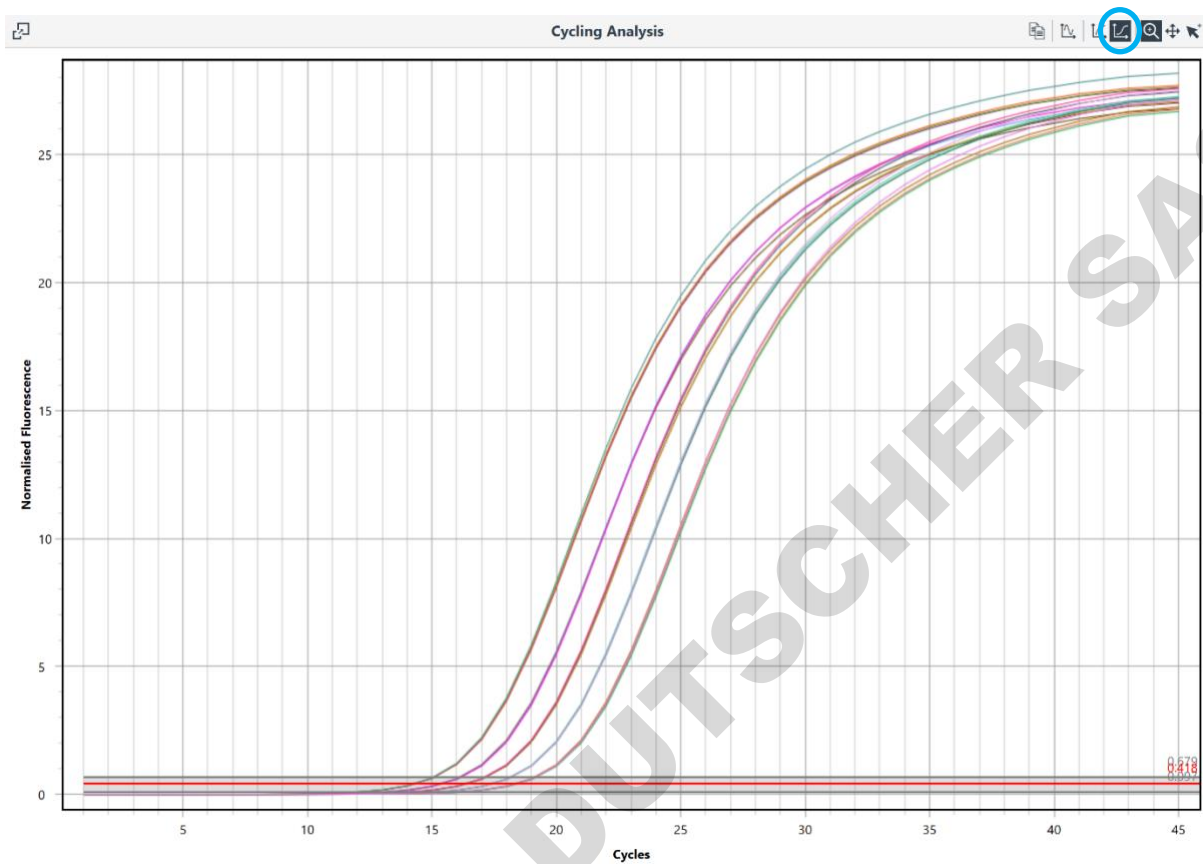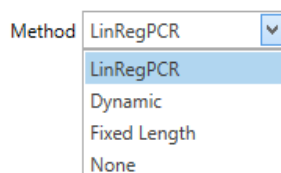
Log *y*-axis: displaying the data in the logarithmic view allows you to better visualise the exponential region of the amplification curve and is therefore the default option. You can revert to the logarithmic scale by selecting the *Log y-axis* icon.

Cycling Analysis Parameters

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:

Method | LinRegPCR |▼|
LinRegPCR
Dynamic
Fixed Length
None

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 cut-off 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*.

Ignore First Cycles [1 ⌄]

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.

Auto set threshold ☑

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.

Auto set threshold ☐ Threshold Level [5.379 ⌄]

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.

Threshold Start 1.00

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%.

Fluorescence Cutoff Enable ☑ Fluorescence Cutoff Level 5.0%

Auto Exclusion: If the sample is excluded its C_q value is not determined and it is not included in the window of linearity calculation. Depending on the exclusion setting a sample may be excluded for one or more of the following reasons:

No amplification: The filtered fluorescence trace for the sample does not show sufficient amplification. The change in fluorescence for the sample must increase by 7 times. The criterion is taken from 'Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data' Nucleic Acids Research, 2009, Vol. 37, No. 6 e45, in the caption for Figure 3. To be included the sample fluorescence must satisfy the following condition

$$f_{first} - f_{min} \geq 7 \times (f_{max} - f_{min})$$

Where;

f_{min} is the smallest filtered fluorescence value;

f_{first} is the first filtered fluorescence value different to f_{min};

f_{max} is the maximum filtered fluorescence value.

Fluorescence Cutoff: The largest value for the filtered fluorescence trace for the sample does not exceed the fluorescence cut-off level and fluorescence cut-off is enabled.

Normalisation failure: The cause of the normalisation failure depends on the type of normalisation being performed:

- LinRegPCR – the iteration limit is exceeded trying to find a satisfactory base line;
- Dynamic – an initial linear region is unable to be found;
- Fixed Length – there are no failure conditions.

The user can set the exclusion to one of three values:

None: no samples will be excluded.

Simple: samples will be excluded if they display no amplification or normalisation fails.

Extensive: samples will be excluded if they display no amplification, if normalisation fails or they fail the fluorescence cut off.

If a sample is excluded its Result column entry of the Cycling Analysis Result table will be set to *Excluded*.

Window of Linearity: Any sample which is excluded will not be included in the window of linearity calculation. In addition, samples may also be excluded from the window of linearity calculation if:

- The second derivative maxima occur at the extreme of the data range;
- The logarithm of the normalised fluorescence trace is not defined near the second derivative maxima.

If a non-excluded sample was excluded from the window of linearity calculation, then it will not have an efficiency or R2 value calculation and its Result column entry of the Cycling Analysis Result table will be set to *Excluded from WOL*.

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:

Well	Cq	Efficiency	R²	Result
▲ **Standard 1**			$\bar{x} = 14.29$	$\sigma = 0.02$
1	14.31	0.91	1.00000	
2	14.29	0.92	1.00000	
3	14.31	0.91	0.99999	
4	14.27	0.92	1.00000	
▲ **Standard 2**			$\bar{x} = 15.37$	$\sigma = 0.03$
5	15.33	0.92	0.99999	
7	15.37	0.93	0.99998	
8	15.40	0.91	0.99998	
▶ **Standard 3**			$\bar{x} = 16.37$	$\sigma = 0.03$
▶ **Standard 4**			$\bar{x} = 17.40$	$\sigma = 0.02$
▶ **Standard 5**			$\bar{x} = 18.38$	$\sigma = 0.03$
▶ **Unknown Sample 2**			$\bar{x} = 16.34$	$\sigma = 0.00$

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.

R²: 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.
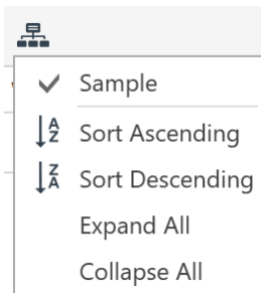
Floating Windows

Under some circumstances it may be necessary to view either the cycling analysis graph or cycling analysis parameters in parallel with another analysis type. For example, you may want to observe what a change in cycle threshold has to reaction efficiency using a standard curve. The floating window option allows you to achieve this multiple analysis window view.

Select the Pop out copy of panel into floating window icon.
Located in the top right-hand corner of either the cycling analysis graph or parameters windows.

You can move the floating window into any position of the screen.
Once the window is floating you can select the parent analysis (e.g. Absolute Quantification).
Any change made to the floating window will be represented in the associated parent analysis.
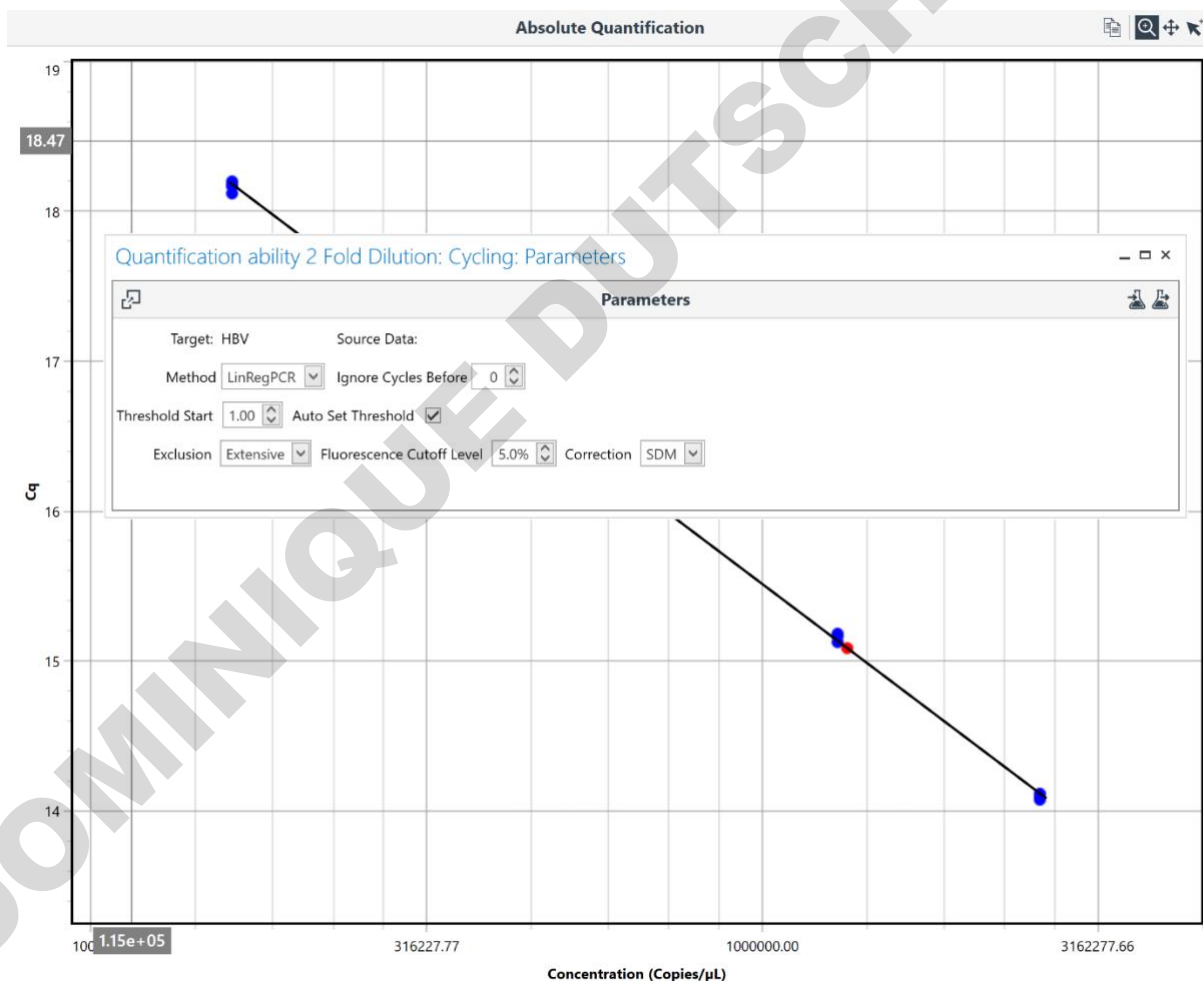
Select the delete icon in the top left corner of the floating window to close the floating window.

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 target that was chosen.

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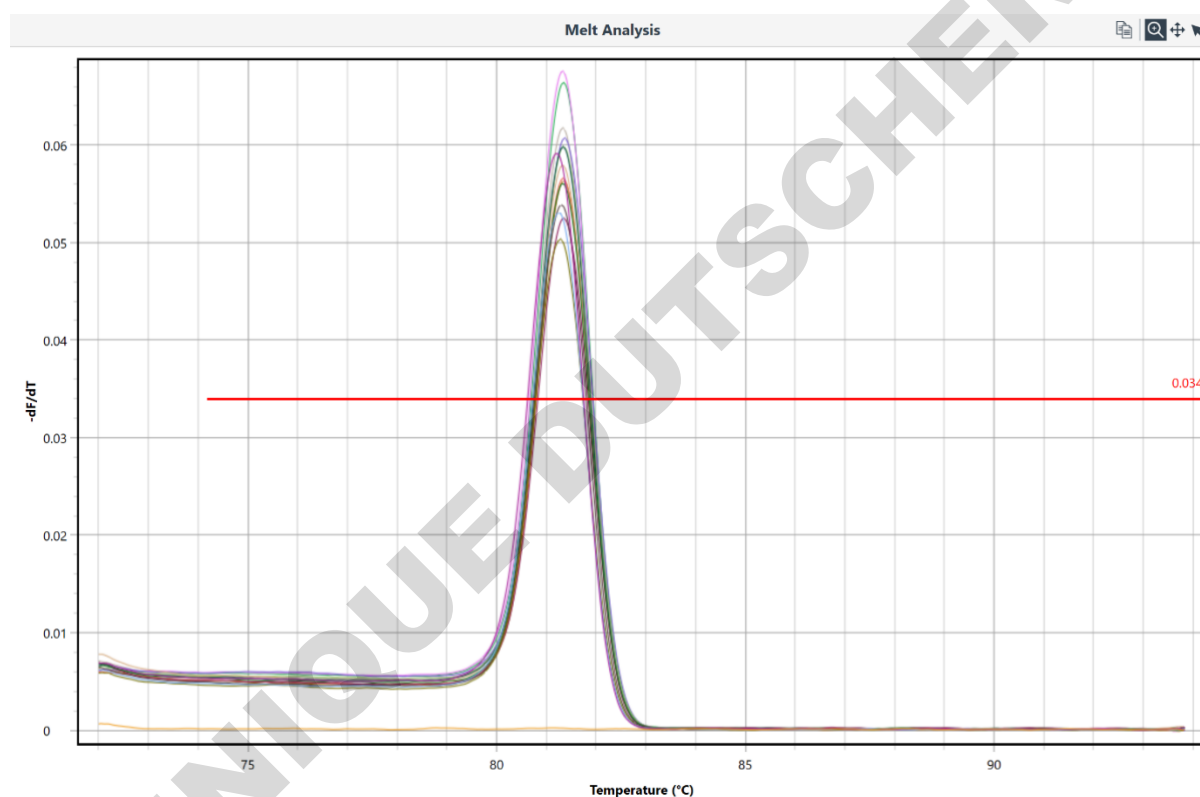
Melt Analysis Parameters

The following parameters can be changed.

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.

Filtering: select the appropriate level of filtering required for the melt data. Options are *None*, *Light*, *Medium*, and *Strong*. The default option is *Medium* and will be appropriate for most assays. For some chemistries such as dual hybridisation probes, a *Strong* filtering option might help improve with melt peak definition.

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.
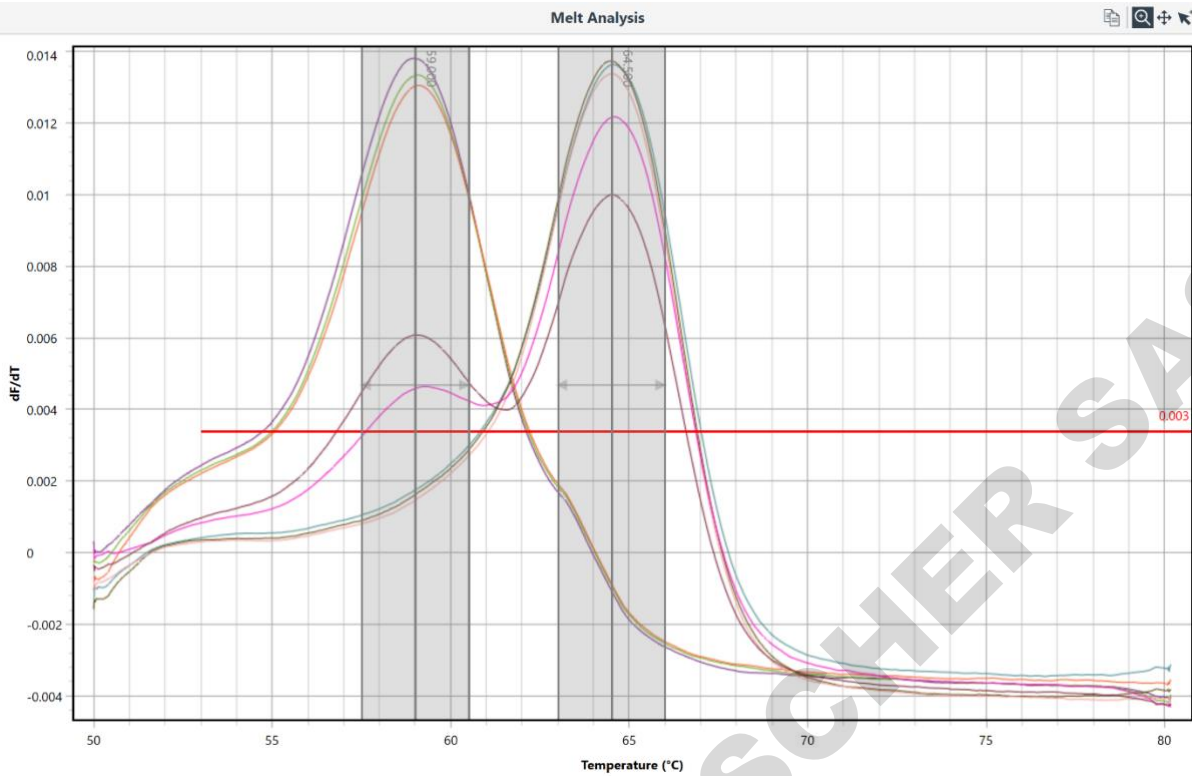
Genotypes

Genotype +	C: 59.00 ⌃⌄ W: 3.00 ⌃⌄	C: 64.50 ⌃⌄ W: 3.00 ⌃⌄	+
Wild Type	✔	☐	✕
Mutation	☐	✔	✕
Heterozygote	✔	✔	✕

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

Well	Sample	Genotype	Tm (°C)
1	Sample 1	Wild Type	58.99
2	Sample 2	Wild Type	59.09
3	Sample 3	Wild Type	59.11
4	Sample 4	Mutation	64.74
5	Sample 5	Mutation	64.68
6	Sample 6	Mutation	64.72
8	Sample 8	Heterozygote	59.19, 64.82
9	Sample 9	Heterozygote	59.05, 64.76

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 Using Absolute Quantification 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. The analysis method is located within the *Absolute Quantification* analysis option.

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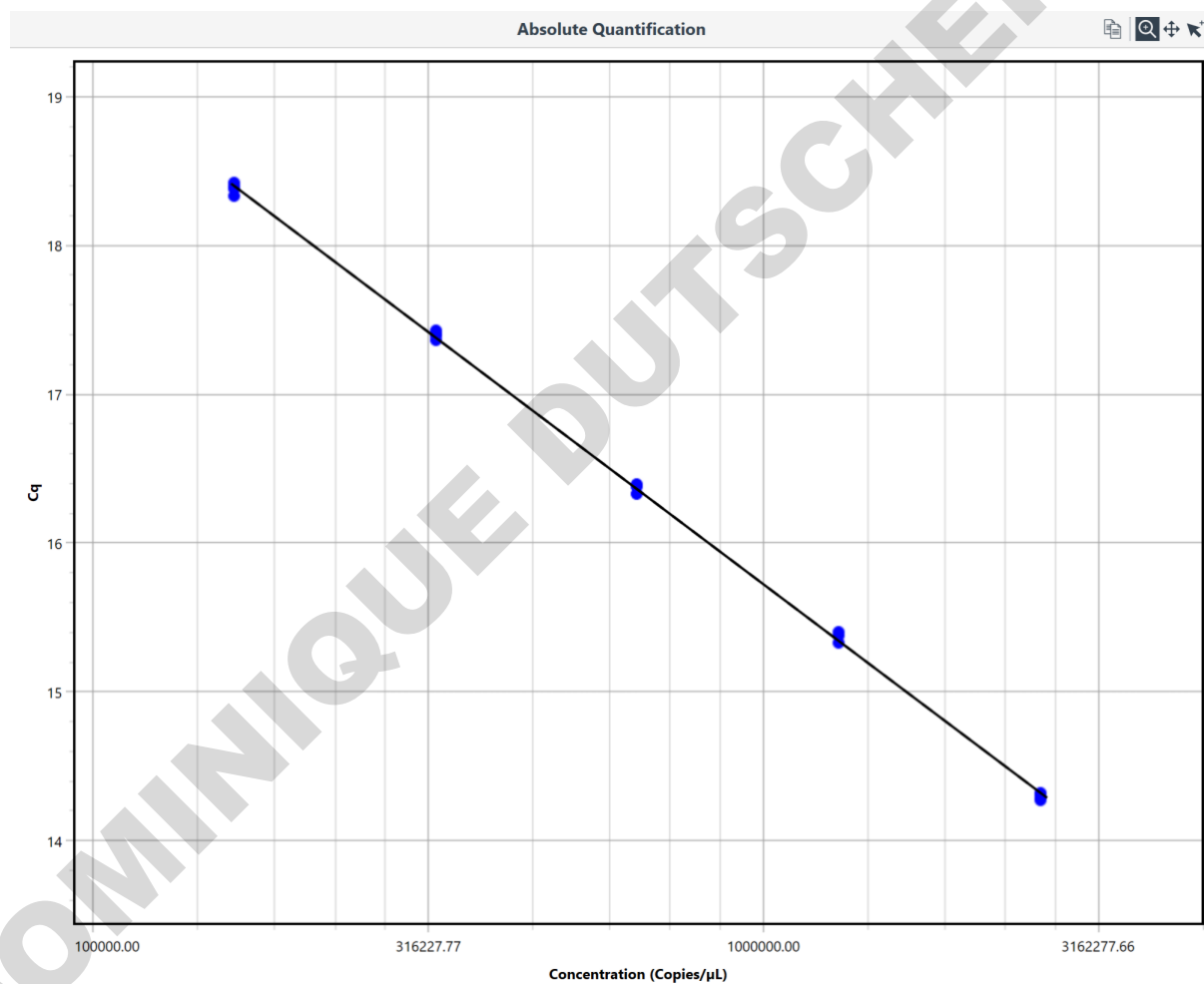
Efficiency: From the slope of the line the efficiency is calculated using the equation $10^{\frac{-1}{Slope}} - 1$, and is reported in a summary window as a value from 0 to 1 along with the *r-squared* (R^2) value, *Equation* of the line, which includes the gradient of the line (*M*), and *y-intercept*.

Standard Curve Characteristics

Equation: y = -3.39 x + 36.06
Efficiency: 0.97 R^2: 0.9995   Export...

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.

Export the standard curve such that it can be used in following runs.
The standard curve is saved into a file location as a *.micsc file. This file can be imported into any run containing cycling data and used as part of the *Absolute Quantification* analysis.

Organise ▾ New folder			
	Name	Date modified	T
Desktop			
Documents	HBV.micsc	30/06/2016 4:09 PM	N
Downloads			
Movies			

File name: Standard Curve.micsc

Save as type: micPCR Standard Curve Files (*.micsc)

Hide Folders   Save   Cancel
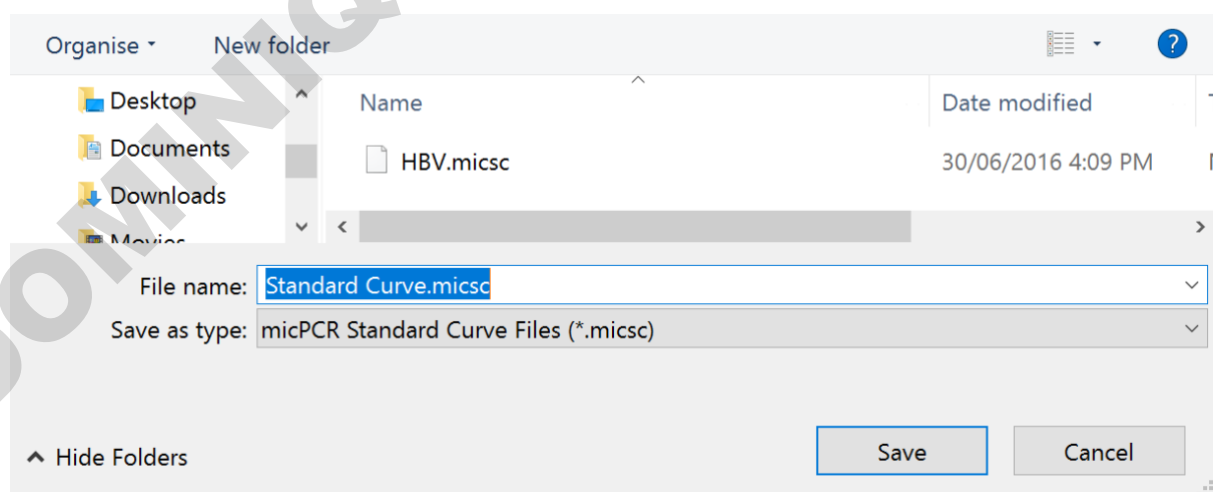
Standard Curve Results Table

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 mean ($\bar{x}$) and standard deviation (σ) 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.

Well	Cq	Given Concentration (Copies/µL)	Calculated Concentration (Copies/µL)	% Variation
Standard Curve Results				
▲ **Sample: Standard 1**			$\bar{x}$ = 2.642E+06 σ = 3.106E+04	
1	14.31	2.6E+06	2.608E+06	0.29
2	14.29	2.6E+06	2.657E+06	2.21
3	14.31	2.6E+06	2.617E+06	0.66
4	14.27	2.6E+06	2.685E+06	3.25
▶ **Sample: Standard 2**			$\bar{x}$ = 1.276E+06 σ = 2.53E+04	
▶ **Sample: Standard 3**			$\bar{x}$ = 6.464E+05 σ = 1.167E+04	
▶ **Sample: Standard 4**			$\bar{x}$ = 3.213E+05 σ = 4886	
▶ **Sample: Standard 5**			$\bar{x}$ = 1.649E+05 σ = 3722	

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.
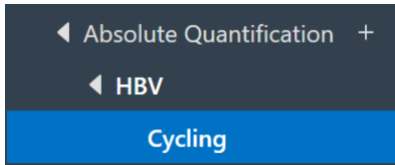
Absolute Quantification

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.
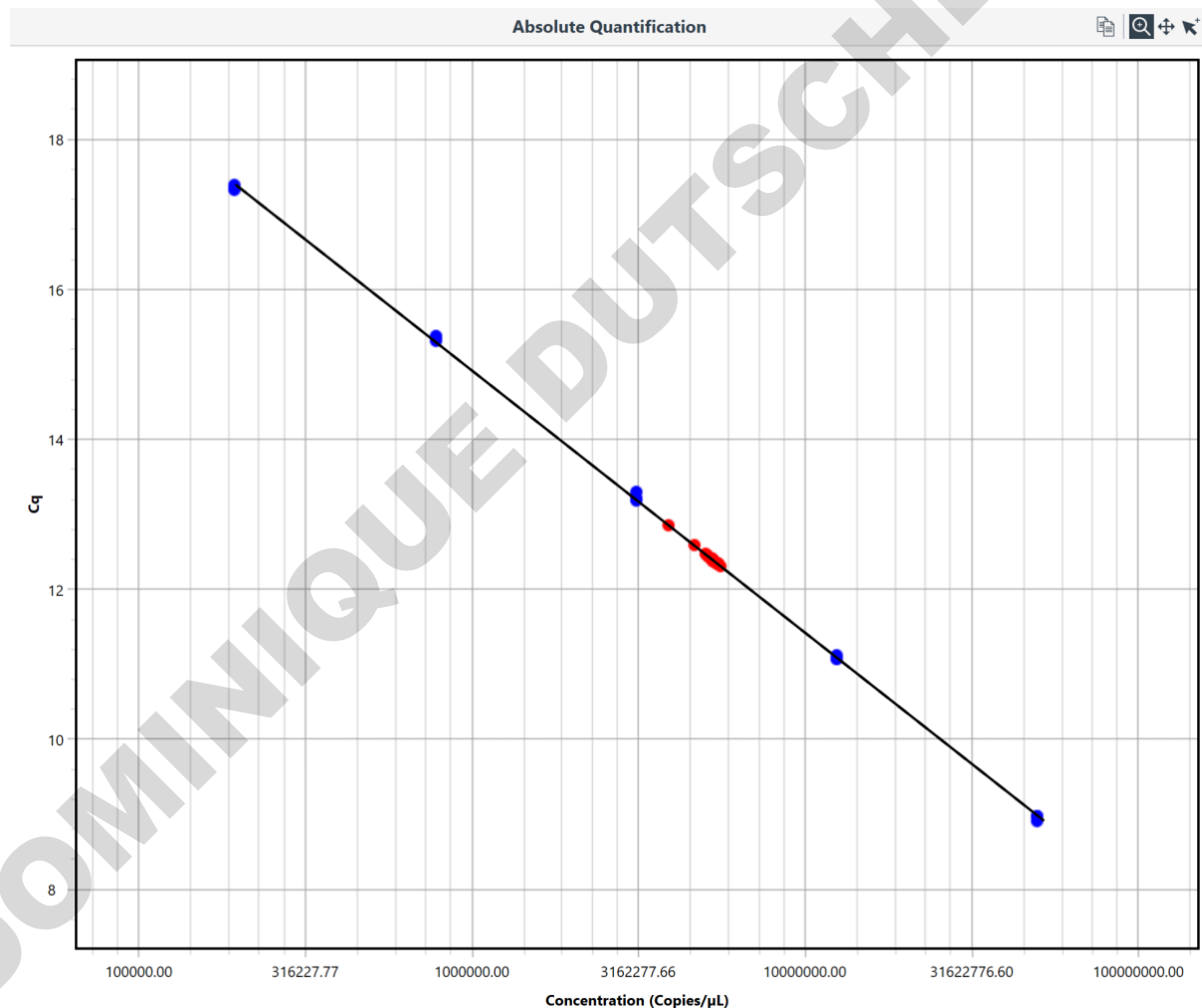
Absolute Quantification Analysis Parameters (Importing Standard Curve)

Import a standard curve.
Locate the standard curve file (*.micsc) in the location the standard curve was exported to. The software will apply the standard curve formula to the run data and display the curve on the graph.
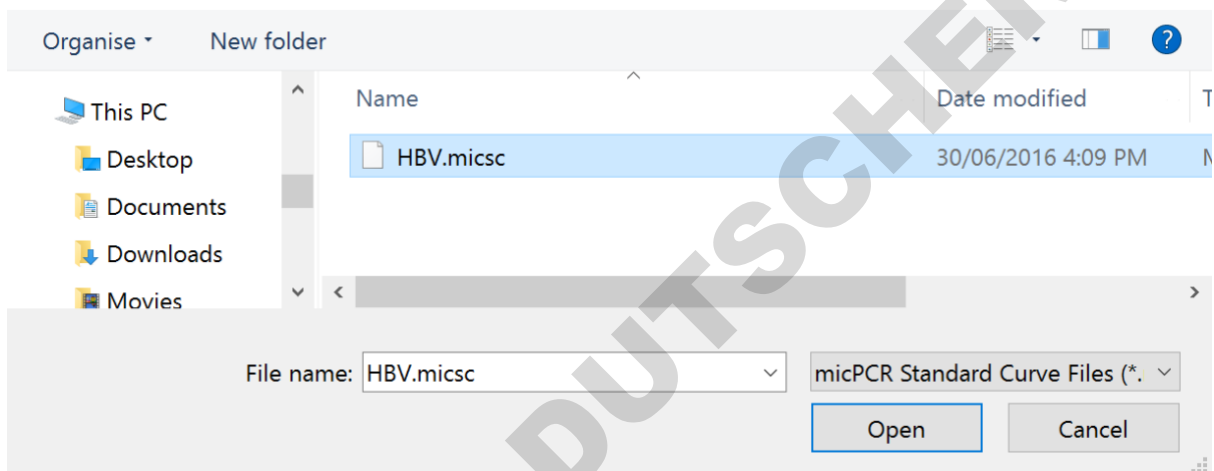
It is required that a standard curve calibrator be used to verify the imported standard curve.
In the Samples editor annotate the calibrator by giving it a Type, Standard. Then enter the concentration for the standard calibrator. A number of replicates can be run for a calibrator but only one calibrator can be applied per imported curve.

Failure to apply a standard calibrator will result in a warning being displayed above the standard curve graph.

A warning will also be displayed if the standard calibrator varies in Cq by more than ± 1 cycles.
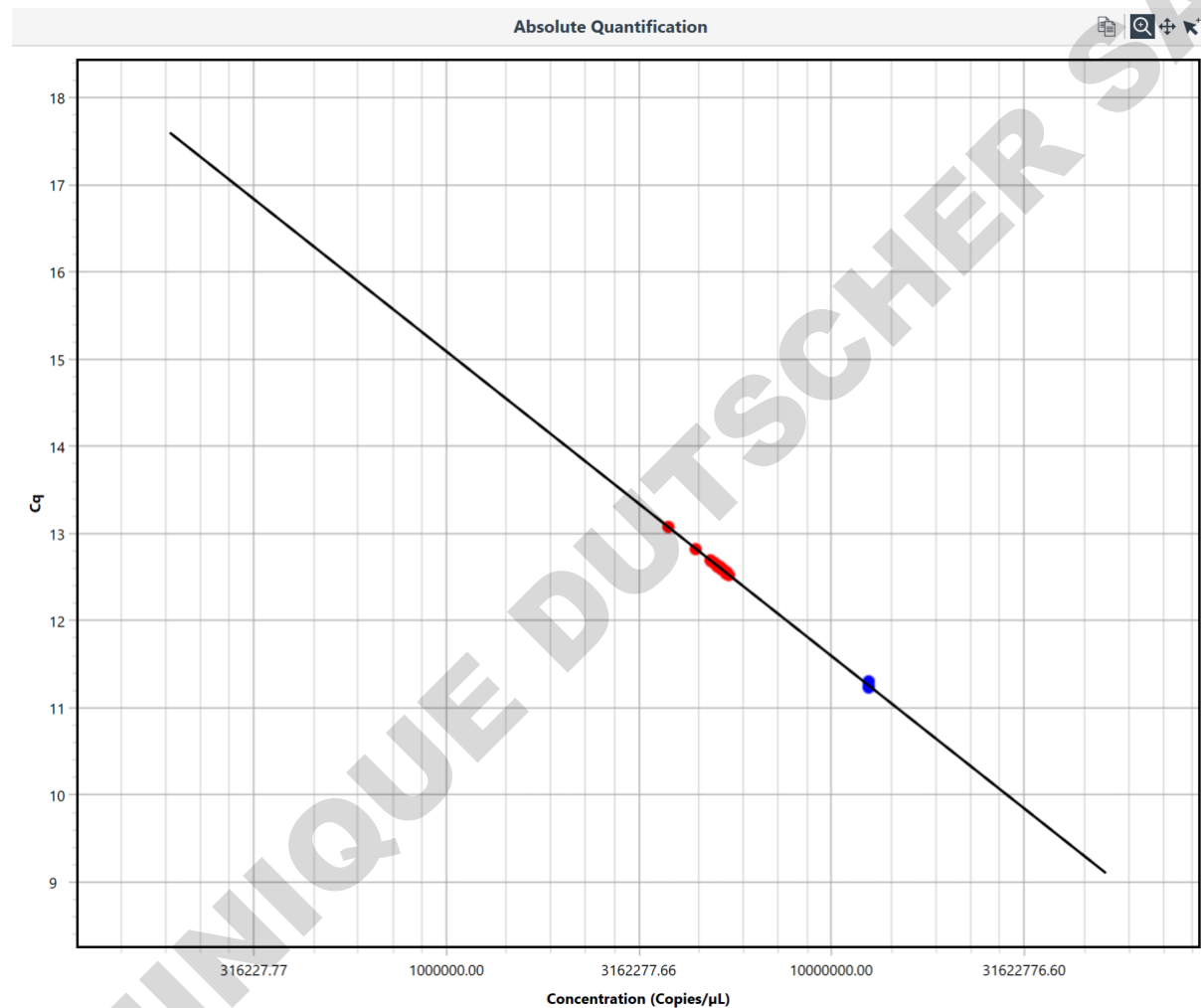
Sample Results Table

The *Sample Results table* reports the *Calculated Concentration* for each unknown sample. The mean and standard deviation 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*.

Well	Cq	Calculated Concentration (Copies/μL)
◢ Sample: Sample 1		$\bar{x}$ = 4.871E+06 σ = 3.056E+05
16	12.59	5.192E+06
17	12.82	4.46E+06
18	12.66	4.961E+06
▸ Sample: Sample 10		$\bar{x}$ = 5.155E+06 σ = 1.3E+05
▸ Sample: Sample 2		$\bar{x}$ = 5.072E+06 σ = 1.439E+05
▸ Sample: Sample 3		$\bar{x}$ = 5.119E+06 σ = 2.133E+04
▸ Sample: Sample 4		$\bar{x}$ = 5.309E+06 σ = 6.851E+04
▸ Sample: Sample 5		$\bar{x}$ = 4.772E+06 σ = 7.034E+05
▸ Sample: Sample 6		$\bar{x}$ = 5.18E+06 σ = 2.214E+05
▸ Sample: Sample 7		$\bar{x}$ = 5.248E+06 σ = 1.089E+05
▸ Sample: Sample 8		$\bar{x}$ = 5.208E+06 σ = 1.224E+05

High Resolution Melt Analysis

HRM will allow you to 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 module that will need to be activated using a key, provided upon sale of the upgrade (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 target that was chosen. 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.
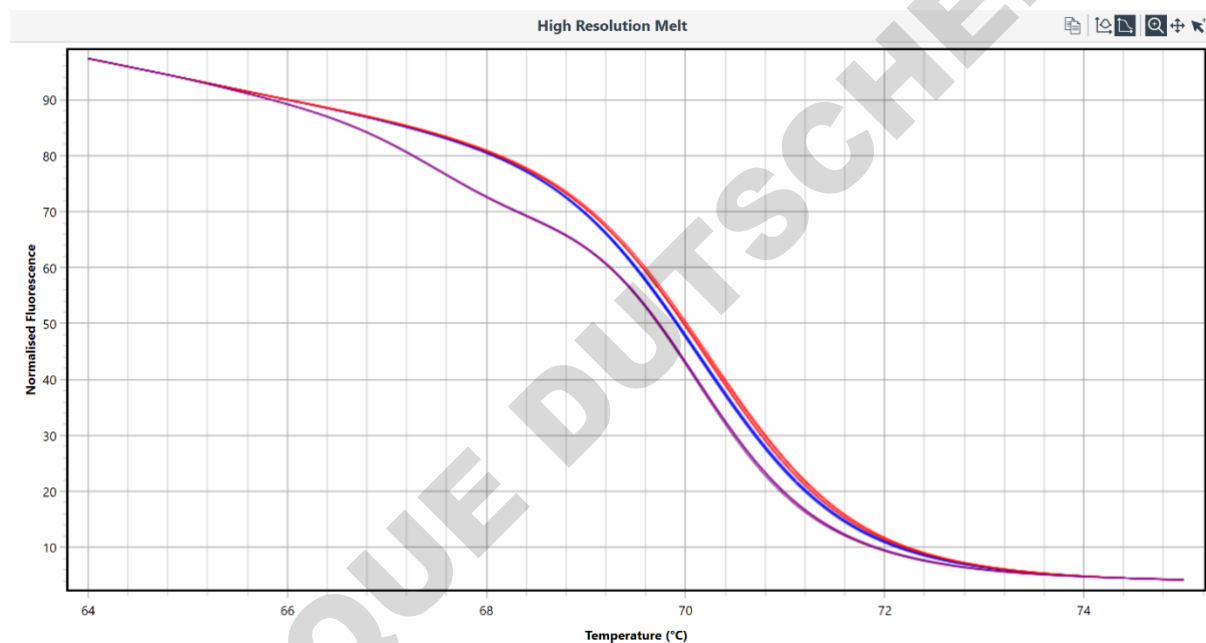
HRM Analysis Parameters

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.
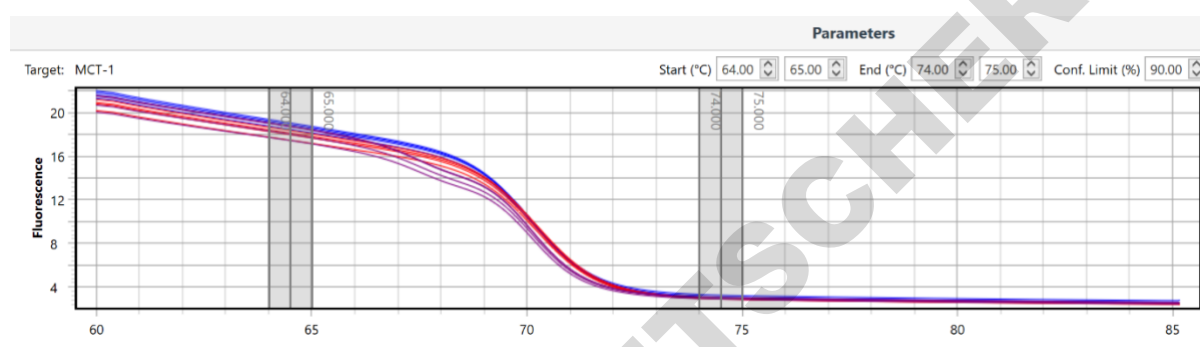
Reference Genotypes

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	WT	∧
5: Mutation Standard	MUT	
9: Heterozygote Standard	HET	
✳		

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.

Set the *Confidence Limit* percentage threshold.

A percentage *Confidence Limit* can be used as an integrity check of the auto-called results. The confidence limit 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.
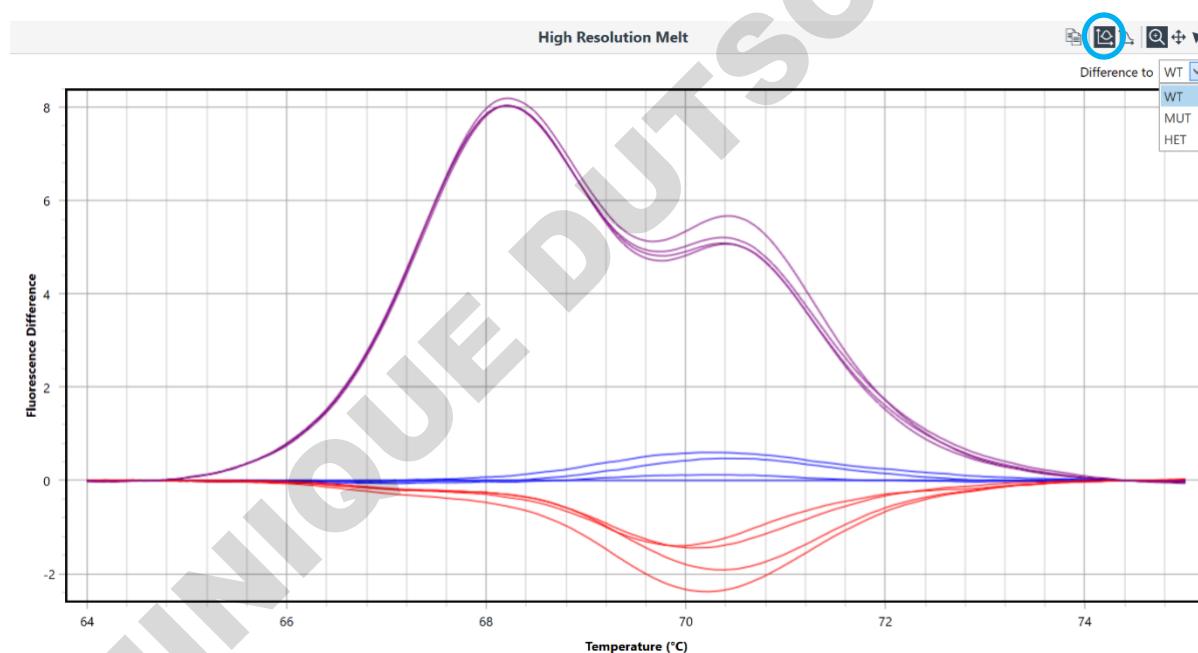
HRM Analysis Results Table

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 limit 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	99.98
3	Sample 2	WT	100.00
4	Sample 3	WT	99.94
5	Mutation Standard	MUT	100.00
6	Sample 4	MUT	100.00
7	Sample 5	MUT	99.47
8	Sample 6	MUT	99.92
9	Heterozygote Stan...	HET	100.00
10	Sample 7	HET	99.96
11	Sample 8	HET	99.97
12	Sample 9	HET	99.99

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 most relative quantification analyses, 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[7, 8]. Furthermore, the efficiency of each gene must be taken into consideration when calculating expression values.

There are three different methods available to achieve relative quantification: REST, ΔΔCt and ΔCt.

REST

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, C_q = 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 C_q 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.

ΔΔCt

We also offer the delta-delta Ct method (Livak and Schmittgen, 2001), which assumes an efficiency value of 1 for each gene used. However, the software does allow the use of individual gene efficiencies in the calculation of relative expression values. In this instance, the same formula described above is used.

---

[7] 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.

[8] micPCR software does not yet apply algorithms to determine the stability of reference genes.

Unlike the REST method, the ΔΔCt method uses a parametric method for its statistical test. A two-tailed student t-test is applied, with the method making the assumption that the data is normally distributed. It is important that you determine this is true for your data sets. If you are unsure, then simply use the REST method described above, which does not make such an assumption.

ΔCt

The delta-Ct method does not apply normalisation of the relative quantification data using a reference gene. It should only be used when the experimental sets are known to be equivalent in starting material.

Relative Quantification Graphs

Gene expression ratios are graphed as either *Box and Whisker plots*, or *Bar charts* depending on the method chosen.

Box and Whisker: Box and whisker plots are only used for the REST method. The box represents the inter-quartile range for the observations, with a median line, and the whiskers are the upper and lower 25% observations from the randomised bootstrapping.
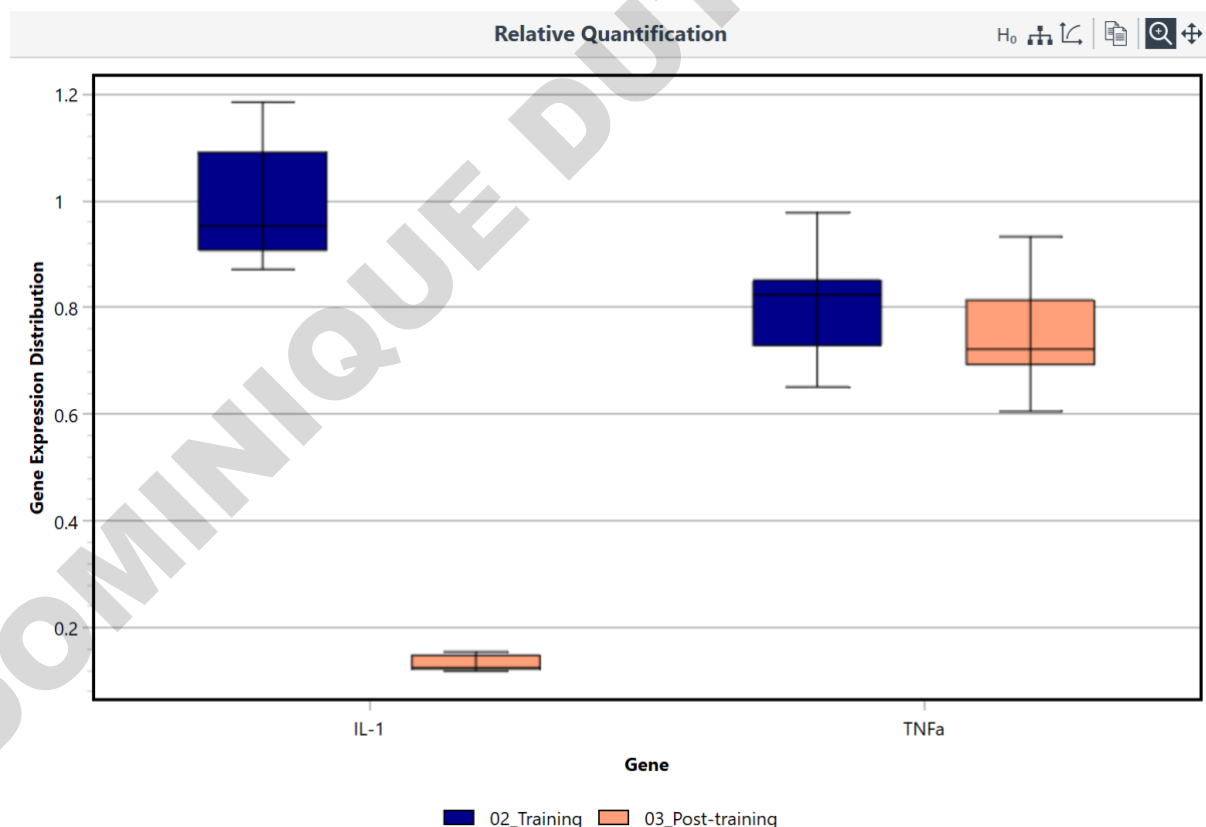
Null Hypothesis Observations: Displays the observations for the random permutations used in calculating the P value for the REST method. Each observation is displayed as an open circle. The calculated mean is shown as a red dot. For a sample to be statistically significant, in relative gene expression, the mean must be toward the outer 5% of observations (up or down). Or to put it another way, there is only 5% or less chance the value is due to randomness.
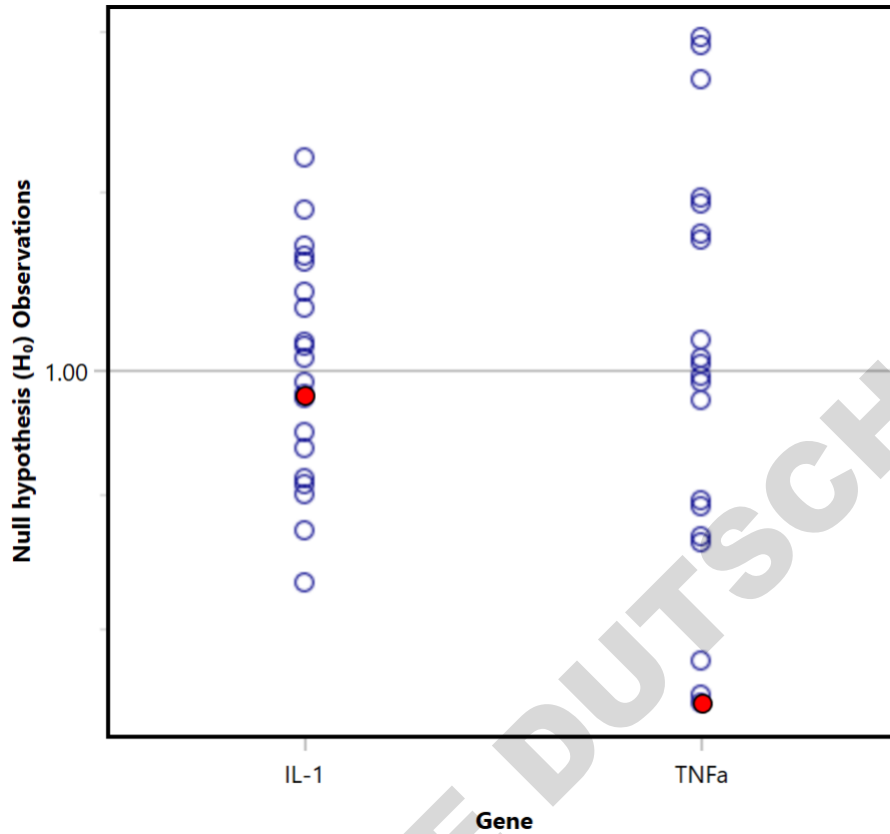
Example Null Hypothesis Observations	In this example, the mean gene expression ratio for the IL-1 gene is within the middle of the observations. Therefore, there is no statistical difference between this group and the control group for IL-1. Conversely, the mean gene expression ratio for the TNFα gene is outside of 95% of the observations. Therefore, there is a statistically significant difference between this group and the control group for TNFα. Further, the position of the mean value is such that the it shows the gene expression is down regulated.

Bar charts: Used for the ∆∆Ct and ∆Ct methods. The mean is shown as the bar and 2-standard deviations are drawn as the error bars.
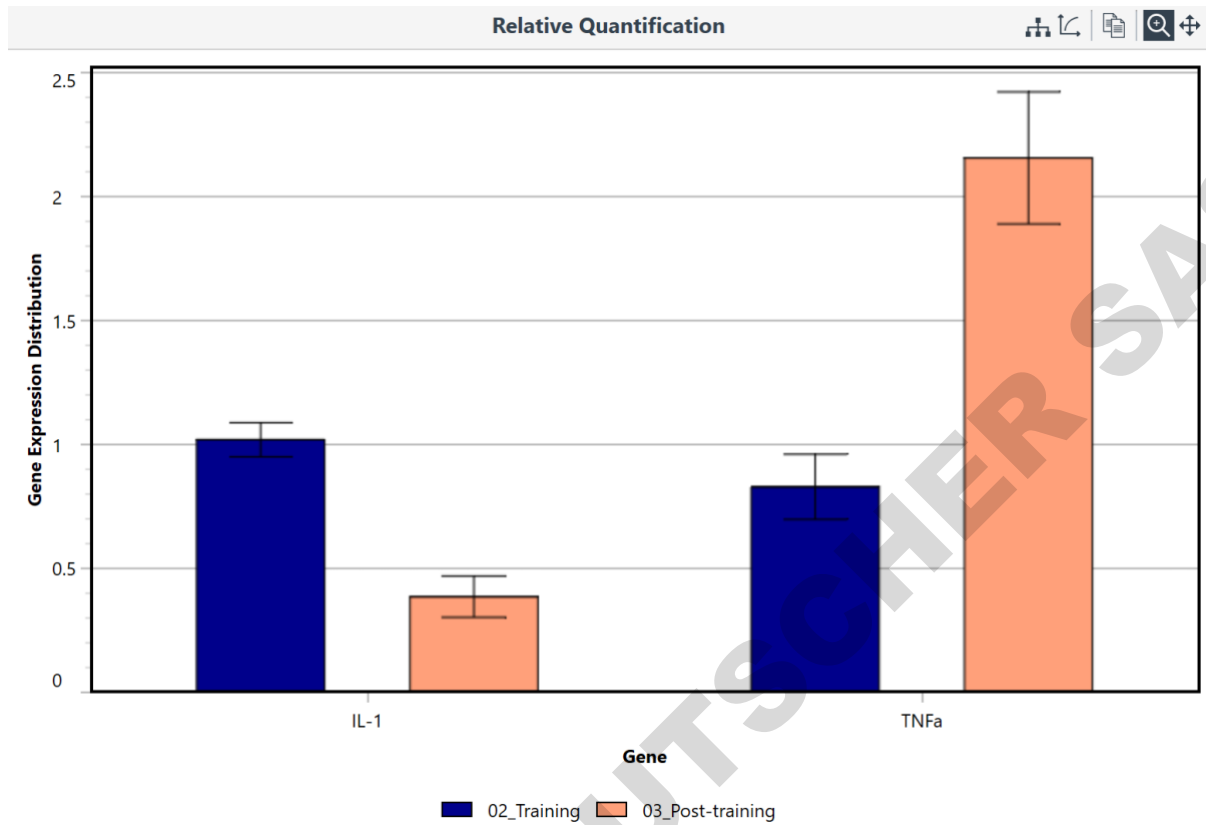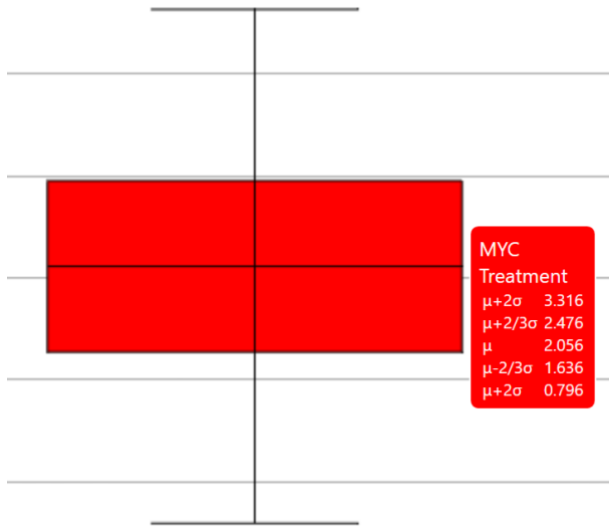
Tool Tip: If you hover over the graphs a tool tip will appear with the numerical values for the box and whisker plot or bar chart.

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.

Log Expression Axis: There is an option to display the gene expression ratio in log scale to allow for better visualisation of bigger differences between groups.
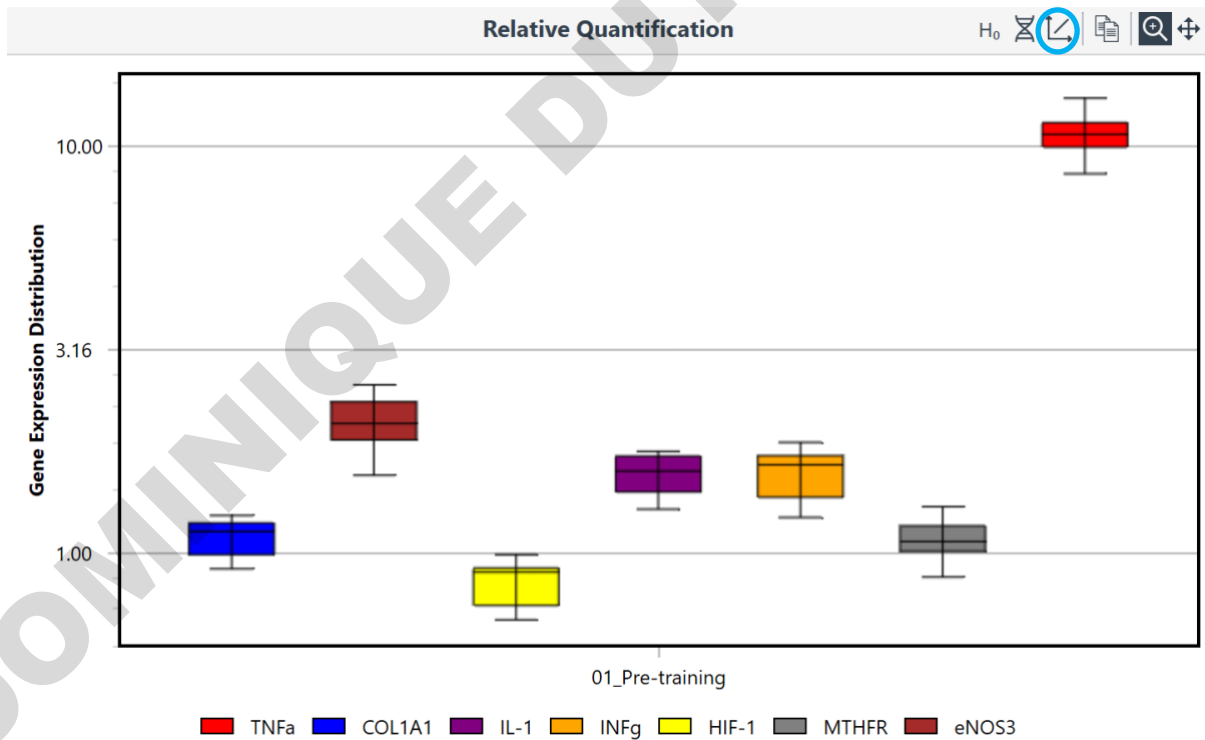
Grouping Function: The graphs can be displayed with either the genes or treatment groups being labelled on the x-axis.

Gene order
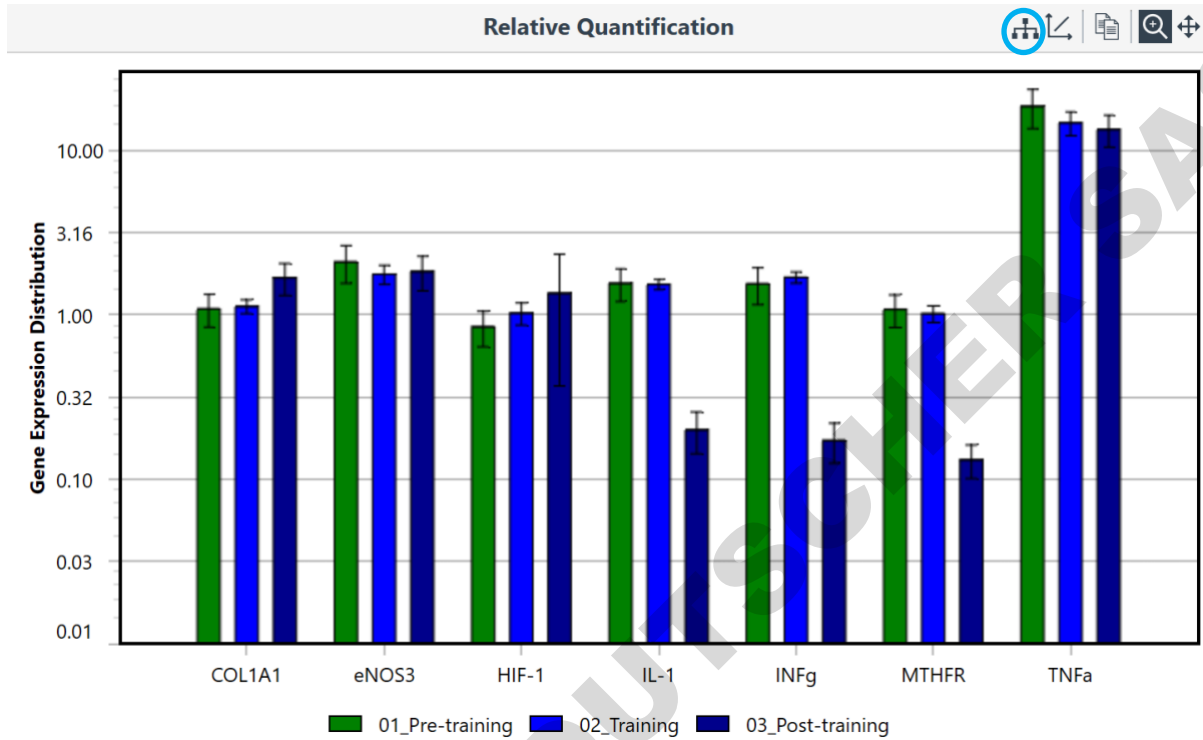
Group order
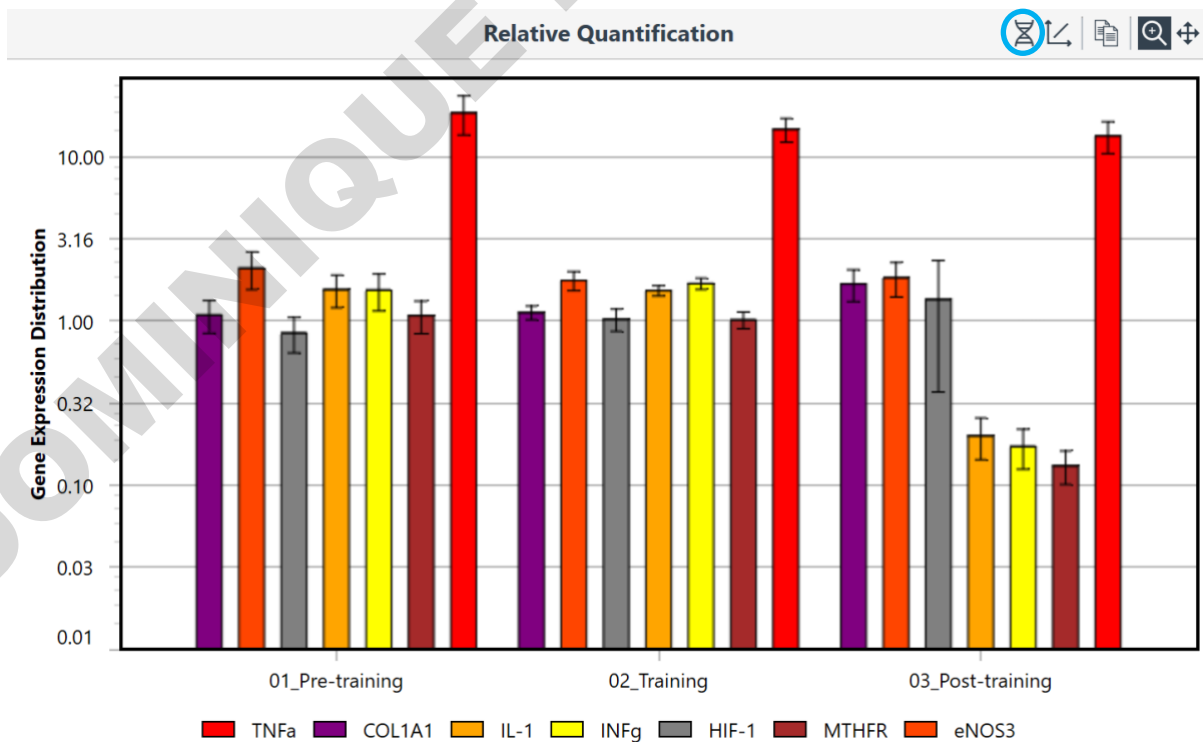
Relative Quantification Analysis Parameters

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

	Gene	Role	Efficiency
🟩	TNFa	Gene Of Interest	1.09
🟪	COL1A1	Gene Of Interest	0.95
🟧	GAPDH	Reference Gene	1.02
🟨	IL-1	Gene Of Interest	1.06
⬛	INFg	Gene Of Interest	1.51
🟥	HIF-1	Gene Of Interest	1.10
🟧	MTHFR	Reference Gene	1.17
🟩	eNOS3	Gene Of Interest	1.19

Method: REST   Efficiency Source: Cycling

	Group	Role
🟦	01_Pre-training	Treatment
🟦	02_Training	Treatment
🟩	03_Post-training	Treatment
🟥	Untreated	Control

Method: select from the choice of *REST, ΔΔCt* and *ΔCt*.

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 use *Absolute Quantification* analysis to determine the efficiency. Use *Absolute Quantification* 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 W-o-L 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 Colour: edit the colour each gene will be drawn in on the graph.

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 Colour: edit the colour the group will be drawn in on the graph.

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.

REST

> **Standard Error and 95% C.I.**: Observation confidence intervals at 68% (equivalent to a single standard error) and 95% (2-standards) calculated using the bootstrapping technique. You must use biological replicates to achieve a meaningful analysis. Technical replicates are considered the same sample and will result in no variation being detected.
>
> **P Value**: The null 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. The number of biological samples per group must be ≥ 3 to achieve analysis.
>
> **Result**: text annotation of gene expression result described as down, up or no difference.

<div align="center">

Results

Gene	Treatment Group	Expression Ratio	Std. Error	95% C.I.	P Value	Result
MYC	Treatment	2.056	1.877 - 2.284	1.714 - 2.546	0.0010	Up

</div>

ΔΔCt and ΔCt

> **SEM**: the standard error of the mean is an estimate of how far the sample mean is likely to deviate from the population mean.
>
> **P Value**: A two-tailed student t-test is applied between the control and treatment group.
>
> **Result**: text annotation of gene expression result described as down, up or no difference.

<div align="center">

Results

Gene	Treatment Group	Expression Ratio	SEM	P Value	Result
MYC	Treatment	2.056	0.199	0.0004	Up

</div>

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.

<div align="center">

Results

Sample Configuration Errors

Sample	Information
Sample 10 - Treatment	Missing gene 'MYC → MYC'.

</div>

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*.

**Samples Selector for Relative Quantification**

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.

Allelic Discrimination

Allelic discrimination allows for the determination of genotypes using real time kinetic data obtained from multiplexed assays using hydrolysis probe chemistry. A differently fluorescent labelled probe is targeted toward a specific allele, such as a single nucleotide polymorphism (SNP), for a given target. The presence of an allele is indicated by a real-time amplification curve within the specific channel corresponding to the probe designed toward it. The presence of both alleles will indicate heterozygosity.

Using the real time kinetic data from each channel, the software combines the data and normalises it to display all the curves on one graph as *Normalised Fluorescence* (*y*-axis) *vs. Cycles* (*x*-axis). Each allele (probe) is represented in the graph with a specific type of point symbol. The type of symbol associated with the allele is shown in the *Genotypes Map*. A threshold is set and samples with curves for each channel that cross the threshold are called positive for the allele they correspond to.
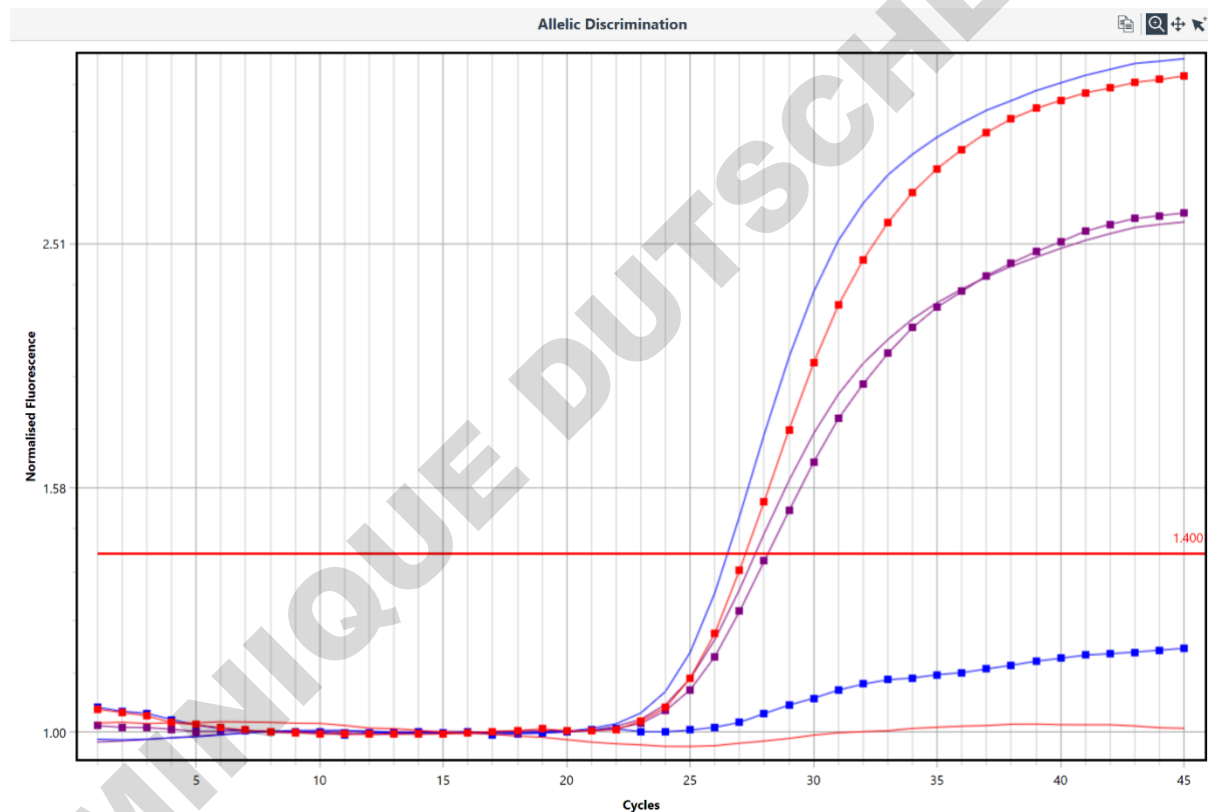
Allele Setup

Each allele will correspond to a specific fluorescently labelled probe, and therefore channel. Using the *Assays Setup*, each allele is defined in the target information page (see Assay Setup for more information).

It is important to select the chemistry type as Hydrolysis Probes or Molecular Beacon.

The *Contains Alleles* check box must be ticked and the name of each allele (probe) entered within the *Probes* name field.
The probe names entered will be displayed in the *Genotypes map* in the *Parameters* settings during analysis.

Finally, it is important to select the corresponding dye for each allele.
If the dye used is not provided in the list, then select an appropriate dye that is similar to the one used. This will link each allele to each channel used.

Complete the remaining steps associated with Assay Setup.

Allelic Discrimination Parameters

Manually set the *Threshold* to a level required to distinguish each allele from background signal levels.

For some assays, minor cross reactivity between probes, cross talk between dyes, or probe self-hydrolysis may result in low levels of kinetic signal for samples that are negative to the allele. Set the threshold above the level of these signals.

The option to ignore the first number of cycles is available along with setting the starting point of the threshold.

	Parameters		
Target: PGM			
Ignore first [5] cycles	Genotype ＋	Wild Type Allele ▬	Mutaiton Allele ■
	Wild Type	☑	☐	✕
Threshold Start [1.00] Threshold Level [1.400]	Mutation	☐	☑	✕
	Heterozygote	☑	☑	✕

Add and name the required number of *Genotypes* for the target.

Link each *Genotype* to one of the alleles displayed in the genotype map.
The allele names displayed are derived from the assay setup.

Allelic Discrimination Results Table

Once the genotype names have been linked to each allele and the threshold is set, the samples are called in the *Results table*.

Results		
Well	Sample	Genotype
1	Sample 1	Heterozygote
3	Sample 2	Wild Type
8	Sample 3	Mutation

Identifier

The *Identifier* software uses a set of rules to auto-call presence or absence of a target(s) in an unknown sample. Controls are also utilised to make appropriate decisions regarding the result of each sample and the run as a whole. These rules can be pre-defined for each target used in the assay individually or as a whole group of targets.

Normalised real-time PCR curves are shown for each target in the analysis graph. Curves are displayed in linear format. You have the option to display all of the targets on the same graph or on individual graphs using the *Chart Display* icons. When the targets are displayed individually, selection of the required target is achieved using the chart tabs.
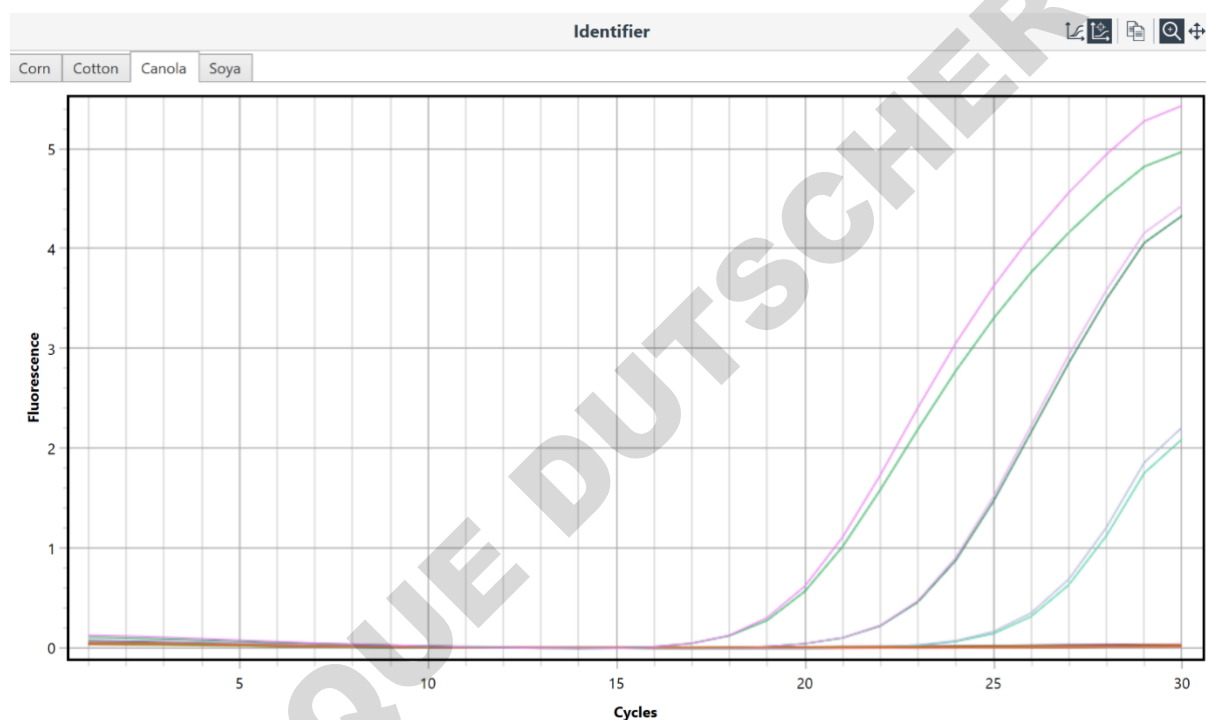
Identifier Parameters

The Identifier analysis uses a logic engine to help automate the identification of a target. The logic engine is a set of rules that are defined by the user that will enable the software to make the identification. The initial rules applied should use various controls within a run to qualify the test.

Setup the required target rules if more than one target is to be used for the Identified analysis.
Use the + icon at the top to add another target set of rules. Give the rule set a name. Each target rule set will be displayed as a tab at the top of the rule sets.

Create the first decision.
Enter a name that will best describe the decision. This name will be displayed in the results table if the rule is met.

When matching the conditions to the data, determine if the rule must meet *All of the following conditions* **or** *Any of the following conditions*.

This option is only valid is using more than one condition to make a decision. For example, if either the target or internal amplification control (IAC), for the positive control, fail to amplify, then use the *Any* option to decide that the run has failed. Since we expect both to amplify in the positive control to pass the test, failure of either one should trigger a failure.

Select the colour of the text for the decision.

Use colours to better differentiate important results. For example, use a red colour to show samples positive for an infectious agent.

Set the conditions for the decision.

> **Target**: select the target required for the rule. A drop-down list will display a list of all targets available for the assay.
> **Sample Type**: includes Unknown sample, Standard, Positive control, Negative control, NTC, or NRT. If using more than one sample in the group type you have the option to select Any or All samples.
> **Quantity**: select the measured quantity as either C_q or *Efficiency*.
> **Comparison**: select from the following options; less than (<), less than or equal to (< =), equal to (=), greater than (>), greater than or equal to (> =), not equal to (! =), *Between*, *Outside*, *Has Value*, or *Has No Value*.
> **Range**: enter a range for the quantity for the comparison. For example, C_q is Outside range of 20 – 30.

Create a new decision using the + Rule button.

You can create as many rules as required. Rules can also be moved by selecting the cross with arrows and dragging the rule up or down the decision order.

The logic engine will use the data from a run to determine a decision for each sample. If the conditions are met for the rule then the decision will be called for the sample or samples. It will call the first rule matched according to the order in the list. If the conditions are not met then the software will continue to the next rule until all the samples are called.

The rules can be reordered by dragging the + *icon*, found to the left of the rule, and dropping on top of another rule to move the dragged rule before the one it is dropped on. Use the x *icon* to delete a rule.

Identifier Rule Guidelines

The analysis works through the rules from the top of the list until it finds a match. Rules after a match is found will be ignored.

The optimum order for specifying rules is

1. Check Controls are OK
2. Check for Positive Decisions
3. All other conditions must be Negative

The following are some simple guidelines to help setup a basic set of rules for a standard presence/absence test.

Positive Controls: Use positive controls to qualify the whole run. Generally, a positive control should amplify during the run for at least the target of interest in the assay. Therefore, use the C_q value of the target of interest and select Has No Value as the rule to fail the run. For some assays, the positive control might be expected to amplify within a certain range. If the C_q falls Outside of this range then the decision would be to fail the run. An example warning might be "Warning: Test Failure".

Decision: Warning - Test Fail | Colour: Black

When matching: ○ All of the following conditions ● Any of the following conditions

Target	Sample Type	Quantity	Comparison	Range	
▶ Turkey	Any Positive Control	Cq	Has No Value		×
IAAC	Any Positive Control	Cq	Has No Value		×
*					×

Negative Controls: we expect a negative control to not amplify for the target of interest. It differs from the NTC in that we do expect the IAC to amplify. We can apply a condition looking for the C_q value of the target of interest to Has Value but we can also apply a condition that sets the IAC to Has No Value. If either of the conditions are met, we fail the test, therefore we need to choose the Any option.

Decision: Warning - Test Fail | Colour: Black

When matching: ○ All of the following conditions ● Any of the following conditions

Target	Sample Type	Quantity	Comparison	Range	
Turkey	Any Negative Control	Cq	Has Value		×
IAAC	Any Negative Control	Cq	Has No Value		×
▶					×

NTC: A no template control is typically used as measure of contamination during PCR setup. We do not expect the NTC for either target to amplify, therefore, the set rule would be to look if the NTC Has Value. However, for some assays the NTC might contain primer dimer and there is provision for an acceptable level. For such tests the condition might be to look for C_q less than a set value. An example decision might be "Warning: Contamination detected".

Decision: Warning - Test Contamination | Colour: Black

When matching: ● All of the following conditions ○ Any of the following conditions

Target	Sample Type	Quantity	Comparison	Range	
▶ Turkey	Any NTC	Cq	Has Value		×
*					×

Positive Sample: For a typical assay, a sample will be called positive for a target of interest if the C_q Has Value. You might choose to use a distinctive colour such as red to highlight such samples.

Decision: Turkey Positive				Colour: Green		
When matching: ● All of the following conditions ○ Any of the following conditions						
Target	Sample Type	Quantity	Comparison	Range	
▸ Turkey	Sample	Cq	Has Value		×
*					×

Negative Sample: Samples that do not meet any of the above rules would be assumed to be absent for the target as long as the IAC Has Value. A positive amplification for the IAC indicates that the absence of the target of interest was not due to a sample amplification failure.

Decision: Turkey Negative				Colour: Red		
When matching: ● All of the following conditions ○ Any of the following conditions						
Target	Sample Type	Quantity	Comparison	Range	
▸ IAAC	Sample	Cq	Has Value		×
*					×

Therefore, a final decision might incorporate a rule that looks for samples where the IAC Has No Value. For such results the decision could be "Warning: Sample Failure".

Decision: Warning: Sample Failure				Colour: Black		
When matching: ● All of the following conditions ○ Any of the following conditions						
Target	Sample Type	Quantity	Comparison	Range	
▸ IAAC	Sample	Cq	Has No Value		×
*					×

Identifier Results Table

The decision for each sample being analysed will be displayed in the results table in the text of the chosen colour of the decision. If multiple rules sets are used these will be displayed in a tab format. Samples will be displayed in well order and each of the controls clearly labelled as type in the *Decision column*. Samples that have been deselected by the user will be reported as such in the decision column.

Results

Corn GMO	Cotton GMO	Canola GMO	Soya GMO

Well	Sample	Decision
1	8	Cotton Natural
2	8	Cotton Natural
3	7	Cotton Natural
4	7	Cotton Natural
5	6	Cotton Natural
6	6	Cotton Natural
7	5	Cotton Natural
8	5	Cotton Natural
9	4	Cotton Natural
10	4	Cotton Natural
11	3	Cotton GMO positive
12	3	Cotton GMO positive
13	2	Cotton GMO positive
14	2	Cotton GMO positive
15	1	Cotton GMO positive
16	1	Cotton GMO positive
17	NTC	NTC
18	NTC	NTC
19	PTC	Positive Control
20	PTC	Positive Control

**Cycling Analysis for Identifier**

Cycling Analysis is used to determine the C_q and Efficiency values for the *Identifier* logic engine. Therefore, *Cycling Analysis* is automatically paired with, and will appear beneath, the *Identifier Analysis* in the navigator bar. Select *Cycling Analysis* to view the cycling analysis parameters and normalised fluorescence curves. Changes to the stand-alone version of cycling analysis will have no effect on the identifier results.

Projects

Projects allow the user to combine multiple runs into one analysis. Up to 10 runs can be combined per project allowing up to 480 samples to be analysed at once.
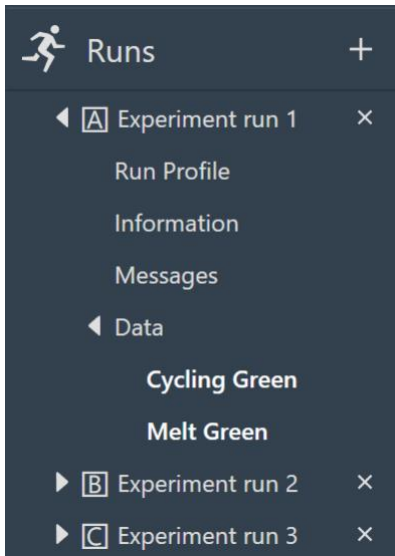

| **NOTE** | The current version (2.0) only allows for Cycling Analysis, Melt Analysis, Absolute Quantification and Relative Quantification. |

To analyse multiple runs, select *New* and then *Projects*.
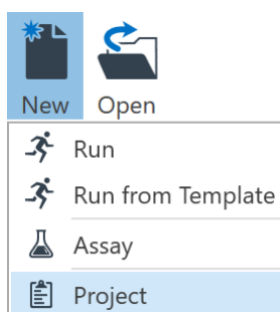
**Select the run files you wish to combine and analyse.**
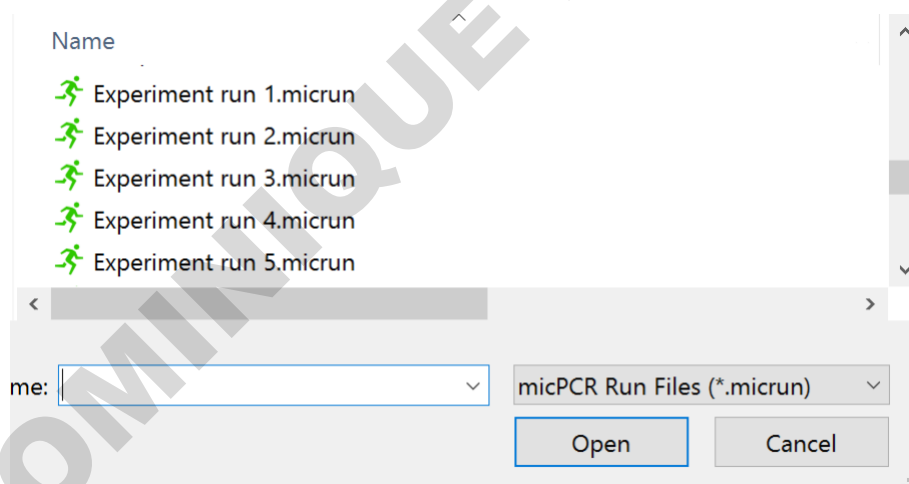
Ensure that the run files are compatible with each other. For example, make sure that the run profiles match.

Each added run file will be labelled alphabetically (A, B, C…) in the order they are selected. Samples from that run will also be labelled with the run prefix in the results table.

Information about each run file, such as *Profile*, *Samples*, and the raw *Data*, can be brought up by opening the run file tree in the Navigator bar.

Remove a run file from the project by using the *Delete* button.
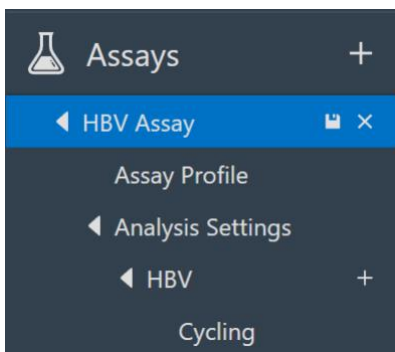
Each Assay that was used in the run set will appear in the *Assay* section of the Navigator bar.

You can look at the *Assay* settings and modify them by opening up the tree.
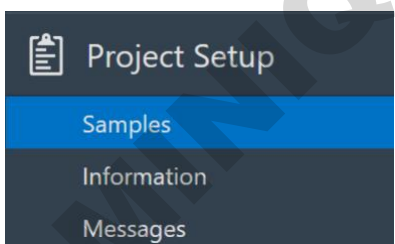
View and/or modify the *Samples Editor* through the *Project Setup* section of the Navigator bar.

Samples are displayed for each run and are listed in the order that the run was selected at the start. The run name is displayed at the top of the sample list and each run list can be collapsed or expanded using the small triangle icon to the left of the run name.

Well-number styles are retained until you select the format in the project sample editor where all runs are reset to that format. The selected button in the sample editor will be set to the option used by the first run, so if you want to change all runs to match, you have to select another option then change back to the one required.

Enter any *Information* about the project in the field provided.

All operations during the analysis of the project will be recorded and displayed in the *Messages* section.

Project Analysis
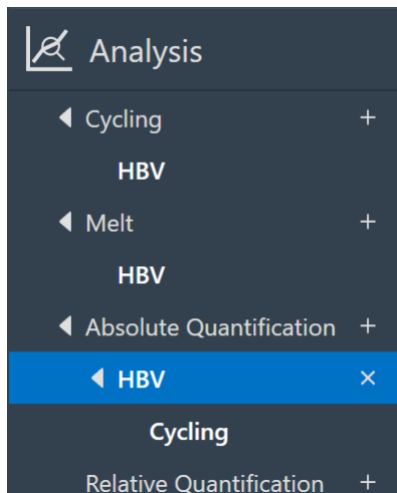
Select the + icon next to analysis type required, to start a new analysis.

Chose the assay you wish to analyse from the list of options provided.

All the samples will be displayed in the one graph.

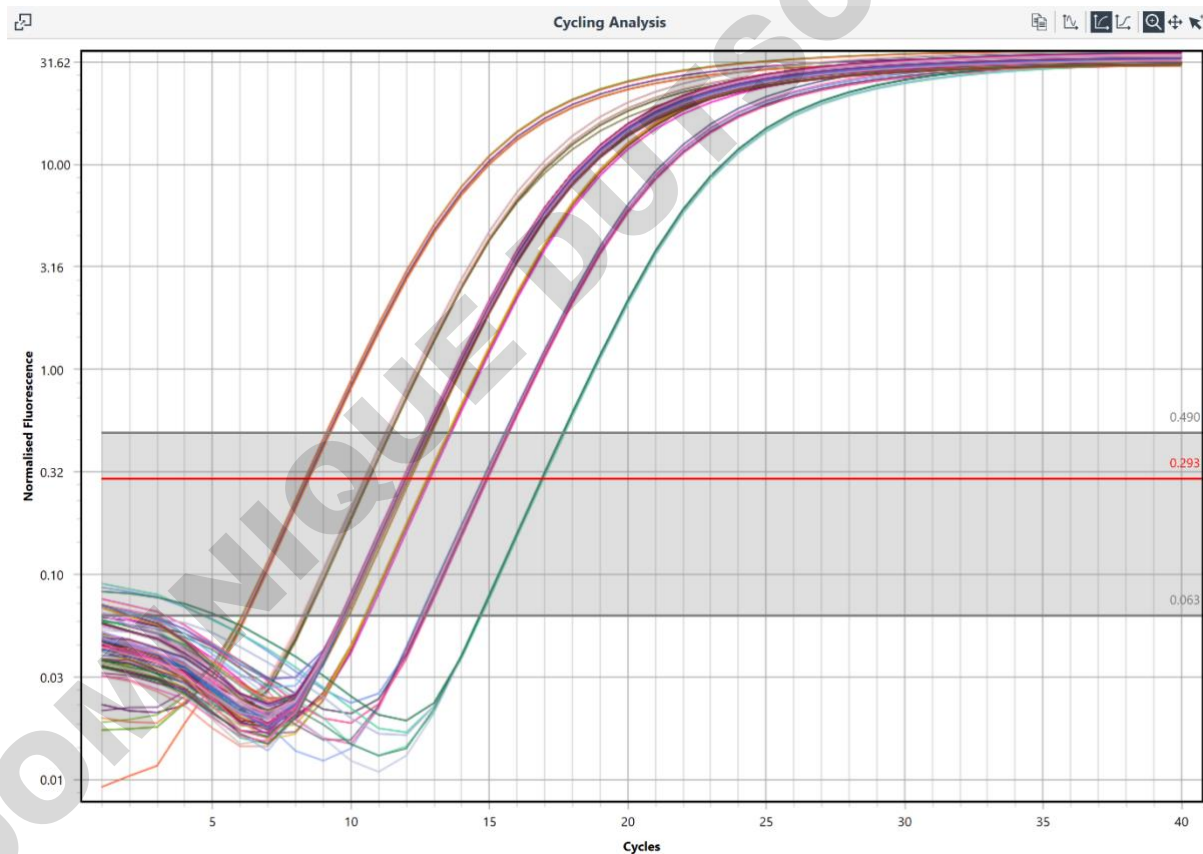All of the same options available for each analysis type are available for a *Project* (see Analysis).

The result tables are similar, however, the results for individual samples are reported with a run order pre-fix for the well (e.g. 1A, 1B, 1C…).

Well	Cq	Efficiency	R²	Result
▸ **NTC**				
▴ **Sample 1**			x̄ = 12.00 σ = 0.07	
16A	11.93	0.99	0.99996	
16B	11.95	0.99	0.99995	
16C	11.95	0.99	0.99995	
17A	11.97	0.98	0.99996	
17B	12.12	0.99	0.99999	
17C	12.12	0.99	0.99999	
18A	12.00	0.98	0.99995	
18B	12.02	0.98	0.99994	
18C	12.02	0.98	0.99994	
19A	11.91	0.98	0.99994	
19B	12.03	0.99	0.99997	
19C	12.03	0.99	0.99997	
▸ **Sample 2**			x̄ = 11.96 σ = 0.02	
▸ **Sample 3**			x̄ = 11.95 σ = 0.06	
▸ **Sample 4**			x̄ = 11.95 σ = 0.07	
▸ **Sample 5**			x̄ = 11.95 σ = 0.05	
▸ **Sample 6**			x̄ = 11.94 σ = 0.04	
▸ **Sample 7**			x̄ = 11.95 σ = 0.04	
▸ **Sample 8**			x̄ = 11.94 σ = 0.06	
▸ **Standard 1**			x̄ = 8.35 σ = 0.06	
▸ **Standard 2**			x̄ = 10.51 σ = 0.06	
▸ **Standard 3**			x̄ = 13.43 σ = 0.97	
▸ **Standard 4**			x̄ = 14.14 σ = 1.03	
▸ **Standard 5**			x̄ = 16.89 σ = 0.03	

The default view for the Samples Selector is divided into runs, with the run name displayed above the list of samples. These can be expanded or collapsed using the small triangle icon.

The *Samples Selector* can also be instructed to show the samples in order of *Assays*, *Sample Groups*, *Sample Name*, or *None* (well order). The groups can also be expanded or collapsed within the Samples Selector using the grouping function.

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Concentration Settings for Projects

Single concentration vs per-channel concentration setting is taken from the first run imported. If concentration units are different, they are changed to "Mixed" and the user has to resolve it themselves.

Well	Cq	Calculated Concentration (Copies/µL)
◢ Sample: Sample 1		x̄ = 4.98E+06 σ = 1.887E+05
17 C	12.33	4.609E+06
18 A	12.20	5.003E+06

Projects can be saved into any file directory.

Amplitude Correction for Cycling Analysis

To correct for variations in signal amplitude for positive samples between runs and instruments, the software uses an amplitude correction algorithm.

To ensure amplitude correction is applied during cycling analysis you must ensure that the same sample is added between different runs.
The sample, called an *Amplitude Corrector*, can be an unknown, standard or positive control. That is any sample that will generate a signal with an observable amplitude. NTC, NRT and negative controls should not be used.
Ensure that the same name in the Sample Editor is used between the different runs.
Multiple samples can be used, but you must ensure a link exists between each of the different runs being analysed as part of a project. For example: Sample 1 could be used between runs A and B, and Sample 95 could be used between runs B and C.

Amplitude correction works by using the First Derivative Maximum to determine a scaling factor between the different runs using the amplitude corrector samples. The scaling factor is then applied to all samples to correct for amplitude differences observed between runs or instruments.

Reports

Generate a report for each *Run* or *Project* using the *Reports +* button in the *Run Navigator*. A standard report will be displayed containing information about *Run Properties*, *Samples*, *Run Profile*, and *Analysis*. A preview of the report will be displayed to the right and can be configured to show only certain parts of the run.

Report Configuration

Each report is divided into three standard sections; *Run Properties*, *Samples*, and *Run Profile*. The remaining sections will depend on the *Analysis* selected in the run. You can choose which sections to display in the report by ticking or unticking the sections in the report *Configuration*.

Report Preview

Each selected section will be displayed in the report *Preview*. A new page will begin following each section. Each page will have a number in page footer along with the version of software used. The run name will be displayed in the page header.

Run Properties

The *Run Properties* section will display the following:

Name	The name of the run
File	The directory location of the run file
Status	
Operator	Name of the individual that completed the run
Notes	Any notes regarding the run completed by the user
Started	Date and time run began
Completed	Date and time run finished
Event Log	A report of important messages generated during the run including any issues such as loss of communication

Samples

The *Samples editor* is replicated in the report preview including sample *Name*, *Type*, standard concentrations, and *Assay*. All 48 samples will be displayed.

Run Profile

All the segments of the run profile are reported for any *Holds*, *Cycling* and *Melts*. The channels used are reported along with the step at which fluorescence was acquired.

Report   *Relative Quantification Run 1*

Run Profile

Hold Steps
Activation at 50°C for 3:00
Hold at 95°C for 5:00

Cycling	
40 cycles	1) 95°C for 15s
	2) 60°C for 30s acquiring on Green

Melt on Green
Melt from 72°C to 95°C at 0.3°C/s

Analysis

Each analysis will be displayed depending on the information provided for the analysis. Only the targets selected will be displayed. To display multiple targets, open up a new analysis for the next targets required. The analysis parameters are displayed at the top of the page followed by a graph of the results. Finally, the results table is reported below the analysis graph.

Report   *Relative Quantification Run 1*

New Relative Quantification

Efficiency	CyclingAnalysisCalculated

Gene of Interest	Efficiency
MYC → MYC	0.97

Reference Gene	Efficiency
B2M → B2M	0.96
ldha → LDHA	0.97

Control Group	Regional
Treatment Groups	Control, Urban

Gene	Treatment	Expr. Ratio	Std. Error	95% Confidence	P Value	Result
MYC → MYC	Control	0.470	0.409 - 0.527	0.377 - 0.564	0.0025	Down
MYC → MYC	Urban	0.935	0.814 - 1.054	0.743 - 1.127	0.2960	NoDifference

Report Options

Search: find a word or string of characters in the report preview. Enter the search word(s) to find them in the report. The located words will be highlighted in the preview.

Print: print the report using user defined settings.

Quick Print: print the report using default print settings.

Page Setup: select the paper type and orientation and adjust page margins.

Zoom: Use the zoom in or zoom out to best view the report preview.

Page selection: navigate through the pages using the page selection buttons; *First page*, *Previous page*, *Next page* and *Last page*.

Export: export the report using one of the available file formats including PDF, XLS and Text file. Each export will have a set of options to choose from.

Send: email a report using one of the available file formats. A report generated in the selected file format will be attached to an email using your default email client, which will open automatically (if available).

Watermark: add a watermark to your report. The water mark can be either a text or image. This option can be used to embed text such as *Confidential* to the report. A list of default text is provided or you can enter your own. Alternatively, add an image to the report such as a company logo. The direction and position of the text or image can be configured as well as which pages to apply the watermark to.

Excel Workbook

Convert a run file into an Excel workbook with worksheets for each section. Do the following to create an excel workbook from your run file:

Click on *Save As* then select *Excel Workbook (*.xlsx)* from the *Save As Type* options.

Once the Excel Workbook is created open it to view the run file.

The workbook will be divided into the following worksheets:
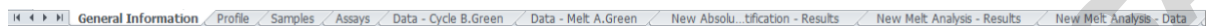
General Information: contains information about the software version, file location, operator, start and finish times along with the run logs.

Profile: information about the run profile is provided including reaction volume and temperature control type.

Samples: the *Samples editor* is reproduced in Excel format.

Assays: information about the chemistry type, target names, reporter dyes and channel used for each target, are shown for each assay run.

Data Cycling: the raw data for cycling is provided for each channel in a separate worksheet.

Data Melt: the raw data for melting is provided for each channel in a separate worksheet.

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Analysis – Results: the analysed results are presented in table format along with various graphs depending on the type of analyses selected in the run file. Each analysis is provided in a separate worksheet.
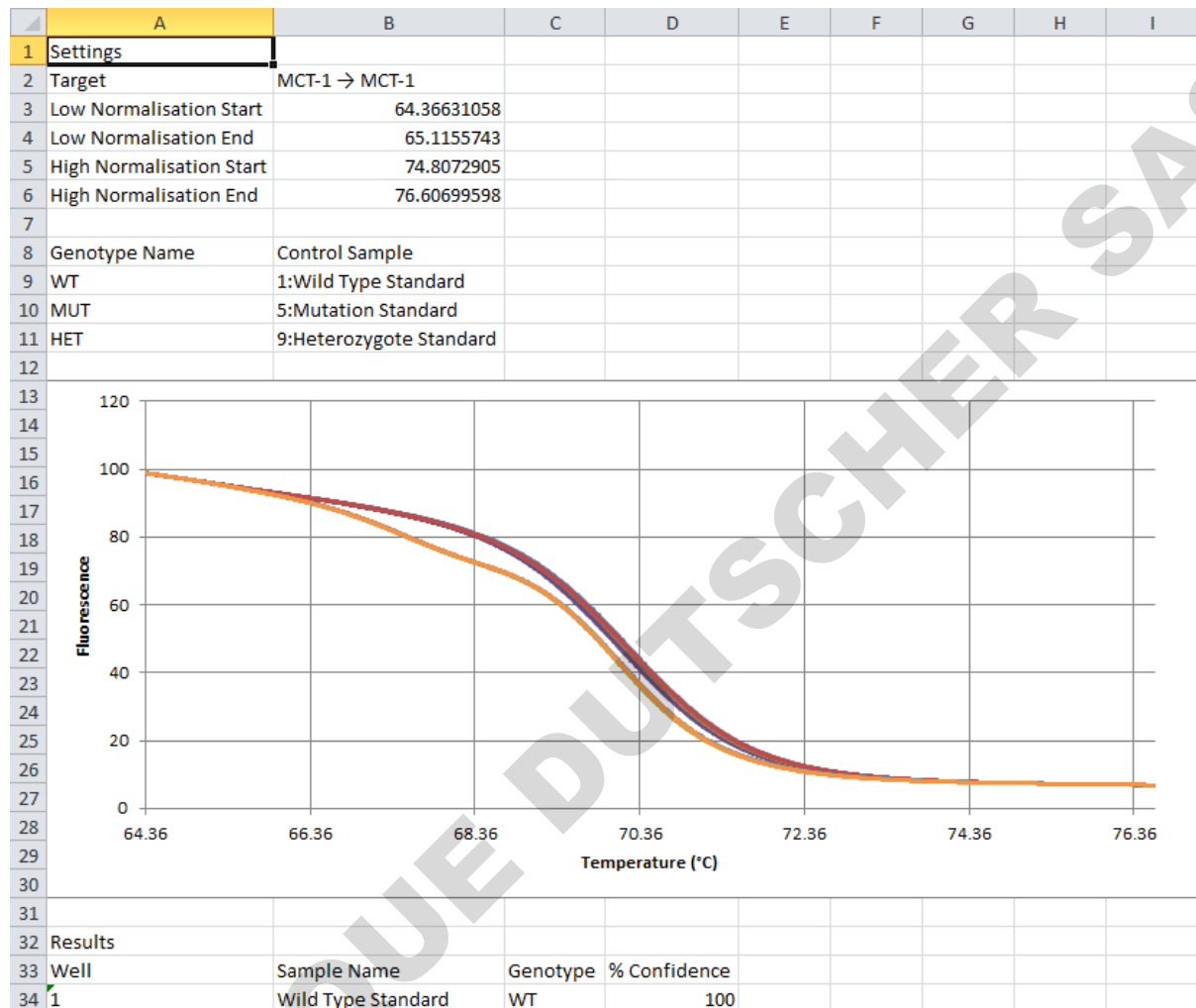
Analysis – Data: the processed data for the analysis is provided (e.g. normalised and filtered melt data).

Laboratory Information Management Systems (LIMS)

It is possible to import sample and assay information from a file generated from a LIMS to the micPCR software for setting up a qPCR run. The micPCR software then controls the instrument to perform the experiment and do the analysis as required. The run information can then be exported from the micPCR software and fed back to the LIMS to archive completed run information.

LIMS Import

The file imported the micPCR software is required to be in either .csv, .tsv or .txt format. Once the file is imported into the micPCR software, a table will display all the fields in the file, where certain fields can be chosen to be imported by linking the column from the files to one of the columns in the sample's editor (i.e. Well, Name, Type, Groups, Assay, Standards Concentration, Input DNA Concentration, Input RNA Concentration, RIN and Lot Number). See *Import Samples* for more detailed explanation.

LIMS Export

The file exported by the micPCR software can be saved as either an excel workbook (.xlsx) or .csv format. The exported file is a multiple worksheet document that is provided in a consistent, systematic format for simple LIMS integration.

The document is composed of the following fixed worksheets:

- General Information about the run (i.e. Date and Time, File Storage, File Status, Operator, Instrument Name and Serial, Firmware Version, User Defined Values (specified on the LIM's configuration page) , Start and End run times)
- Profile information (i.e. Temperature Control, Reaction Volume and Logical File)
- Sample information (i.e. Well, Name, Type, Groups, Assay, Standards Concentration, Input DNA Concentration, Input RNA Concentration, RIN and Lot number)
- Assay information (i.e. Assay Name and Type, Target Name, Probe Name, Type of Reporter and Channel Required for Acquisition)

The document can also be composed of the following non-fixed worksheets (will only be produced if the data is present):

- Raw Data (i.e. A separate worksheet for each channel on which cycling and melt data was acquired)
- Analysis (i.e. A separate worksheet for each analysis created, such as Cycling, Melt, Absolute Quantification, Relative Quantification, Allelic Discrimination, HRM, Identifier Analysis)

LIMS Auto Export

It is possible to setup connection to a Laboratory Information Management System (LIMS) that will allow all data from micPCR run files to be exported into CSV and XLSX formats. You will find this option in the Information page. The file exported will be provided in the same format as when done manually (see *LIMS Export*).
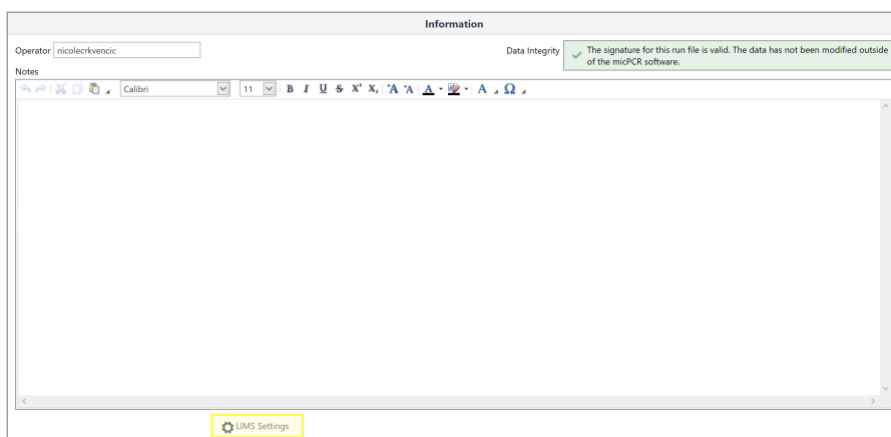
Each micPCR run file contains its own set of key and value meta data that can be user defined.

Enter the required Key labels and their Values in the table. Any user defined key and value combinations will be stored in the run file and exported into the export file.
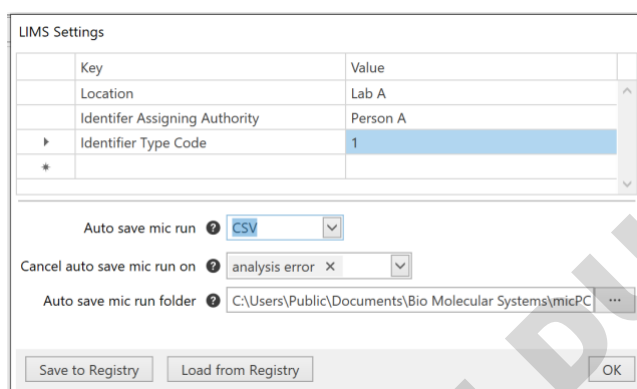
It is possible to automatically save the data from the MicPCR run to a folder that is linked to a LIMS by clicking on the *more* icon (three horizontal dots) and selecting the desired folder. The run data can be auto exported in either *CSV* or *XLSX* format by selecting it in the drop-down menu of *Auto save mic run*.

There is also the option to have the auto export be cancelled if an error is detected in any auto generated analysis. If an error is detected a warning should be displayed allowing you to possible rectify or otherwise process the run file. This can be done by selecting one of the following options in the *auto save mic run* menu: *Analysis error*, *Optical Failure*, *Reportable Warning*, *Run Failure*.

There is also a common LIMS metadata registry used by both micPCR and Myra software which is stored on the computer.

Save to Registry will overwrite the values in the common LIMS setting registry file from the run.

Load from Registry will overwrite the values in the run from the common LIMS setting registry file.

When a new micPCR file is created using *New* it will load the values from the common LIMS setting registry file. When a new micPCR or Myra file is created from a *Template* it will use the meta data values stored in the template. It will not load the values from the common LIMS setting registry file.

Appendix A

Temperature Verification System (TVS)

The TVS can be used to ensure that the Mic is operating to specification in regard to temperature control.

CAUTION

⚠️

TVS sensor is fragile and connected via a very thin cable
Please handle with care to avoid breakage. Hold the device only by the sides.

Verification Environment

For optimal results, it is best to perform the tests away from sources of airflow, for example fans and air vents. The ambient temperature may also have an effect on the measurement. We recommend to test under standard lab conditions (22 – 28°C).

Hardware and Software Installation

Connect the Mic to be verified to your computer and switch it on.

Take the TVS out of the protective case and connect it to your computer through USB.

Open the micPCR software.
Version must be at least 2.6.0.

Verification Process

Once the Mic and TVS are connected to your computer, scan for devices by clicking on the Communication icon.

Select the instrument to test, and select Temperature Verification from the drop-down menu.

Follow the prompts on the TVS Wizard.
During these steps, keep the TVS placed beside your Mic instrument.

When prompted by the software, remove the sensor from the foam slot and place it in wells 1 – 4 with the tab facing inwards.
Keep all other wells empty. Once the sensor is secured in with the tube clamp, close the lid and continue following the prompts on the screen. Verification may take up to 25 min.

At the end, you will be given the option to save or print your verification report.

MIC Temperature Verification Report

Date Completed: 23/05/2017

Instrument: M0000002
Verified By: TVS00006

	Verification Check		
	Ring Temp. (°C)	Actual Temp. (°C)	Measurement Error (Max±0.25°C)
Pt 60°C	60.01	60	0.01
Pt 95°C	95.03	94.97	0.06

This machine has been verified to be working according to specification

_____
Name

_____
Signature

Carefully remove the sensor from the instrument and place it back into the foam slot.
Unplug the TVS from the computer and place it back into the protective case.

Appendix B

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

Appendix C

CFR 21, Part 11

The Code of Federal Regulations Tile 21 part 11 (21CFR11) of the U.S Food and Drug Administration, covers the regulations on electronic records and electronic signatures to ensure trustworthy, reliable and equivalent to paper records. We have implemented a number of features into our software that cover some of the requirements of 21CFR11 section 11.10 for closed systems. The procedures covered are listed and described below. The remaining procedures not covered by us, have a valid reason for each described below.

21CFR11 (section 11.10) states:

"Persons who use closed systems to create, modify, maintain, or transmit electronic records shall employ procedures and controls designed to ensure the authenticity, integrity, and, when appropriate, the confidentiality of electronic records, and to ensure that the signer cannot readily repudiate the signed record as not genuine. Such procedures and controls shall include the following":

"(a) Validation of systems to ensure accuracy, reliability, consistent intended performance, and the ability to discern invalid or altered records".

The software contains run file signatures that ensure validity of the records. Any tampering of the run file through external software will break the signature, resulting in a notification by the software. The Data Integrity can be found in the Information page of the run file.

"(b) The ability to generate accurate and complete copies of records in both human readable and electronic form suitable for inspection, review, and copying by the agency".

This control is met through the generation of pdf reports (see Reports for more information).

"(e) Use of secure, computer-generated, time-stamped audit trails to independently record the date and time of operator entries and actions that create, modify, or delete electronic records. Record changes shall not obscure previously recorded information. Such audit trail documentation shall be retained for a period at least as long as that required for the subject electronic records and shall be available for agency review and copying".

The following will be logged and will appear in the Messages page of the application and the Event log section of the report or Excel® report when the run is saved or when a report is switched to:

- Changes to all parameters of analyses since the log update for analyses created before the last log update

- Changes from default parameter values for analyses created since the last log update

- Removal of analyses

- Changes in Analysis sample selection

There will be a single entry per analysis in the Message panel summarising the changes. The time stamp of the entry is the time of the '*Run Save*' not of the analysis parameter change.

The remaining procedures we have no control over, especially as some of them relate to the whole standard operating procedure for a test and/or quality management process, not just to the software. These include:

"(c) Protection of records to enable their accurate and ready retrieval throughout the records retention period".

Provided by organisation document management system (see note below).

"(d) Limiting system access to authorized individuals".

Provided by organisation document management system (see note below).

"(f) Use of operational system checks to enforce permitted sequencing of steps and events, as appropriate".

This is an organisation operational requirement.

"(g) Use of authority checks to ensure that only authorized individuals can use the system, electronically sign a record, access the operation or computer system input or output device, alter a record, or perform the operation at hand".

Provided by organisation document management system (see note below).

"(h) Use of device (e.g. terminal) checks to determine, as appropriate, the validity of the source of data input or operational instruction".

This is an organisation operational requirement.

"(i) Determination that persons who develop, maintain, or use electronic record/electronic signature systems have the education, training, and experience to perform their assigned tasks".

This is an organisation operational requirement.

"(j) The establishment of, and adherence to, written policies that hold individuals accountable and responsible for actions initiated under their electronic signatures, in order to deter record and signature falsification".

This is an organisation operational requirement.

"(k) Use of appropriate controls over systems documentation including:

(1) Adequate controls over the distribution of, access to, and use of documentation for system operation and maintenance.

(2) Revision and change control procedures to maintain an audit trail that documents time-sequenced development and modification of systems documentation".

This is an organisation operational requirement.

File management is up to the end user. They must configure appropriate permissions and file storage such that it meets these requirements. Windows allows you to have separate permissions to create files or write data and delete. However, it does not distinguish between writing a file and overwriting a file so there is nothing to prevent you from saving one file over the top of another.

It would be our recommendation to use a proper enterprise quality management software system like MasterControl™ (MasterControl Inc., Utah, USA) to meet all the 21CFR11 requirements.

Appendix D

Dye Colour Chart

Dye	Excitation	Emission	Channel	Application
BEBO	468	492		Intercalating
LC Green®	455	495		HRM dye
SYTO® 9	483	503		HRM dye
FAM™ (best)	494	515		Conjugated label
SYBR® Green I	494	521		Intercalating
RiboGreen®	500	520		RNA label
PicoGreen®	502	523		ds DNA label
Eva Green®	503	527		HRM dye
TET™	521	536	suboptimal	Conjugated label
CAL Fluor® Gold 540	522	541	suboptimal	Conjugated label
JOE™	520	548	suboptimal	Conjugated label
VIC®	538	554		Conjugated label
HEX™	535	555		Conjugated label
CAL Fluor Orange 560 (best)	540	561		Conjugated label
Quasar® 570	548	566		Conjugated label
Cy™3	550	570		Conjugated label
NED™	546	575		Conjugated label
TAMRA™	555	576		Conjugated label
CAL Fluor® Red 590	565	588	x	Conjugated label
ROX™	573	602		Conjugated label
Texas Red®	583	603		Conjugated label
CAL Fluor® Red 610 (best)	590	610		Conjugated label
LC® Red 640	620	635	suboptimal	Conjugated label
Quasar® 670 (best)	647	667		Conjugated label
Cy™ 5	651	674		Conjugated label
Cy™5.5	675	694		Conjugated label
Quasar® 705	690	705	x	Conjugated label

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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 probe 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.

Magninic 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.