TECHNE

PrimeQ

OPERATOR'S MANUAL

Bibby Scientific

Version 2.1
08/12

How to use this guide

Please read all the information in this guide before using the unit.

Le rogamos lea cuidadosamente la información contenida en este folleto antes de manipular el aparato.

Veuillez lire attentivement toutes les instructions de ce document avant d'utiliser l'appareil.

This operator guide provides the basic information to get you started on the PrimeQ real-time PCR system. It covers everything from taking the instrument out of the box, installing the application software and system set-up, to setting up, running and analysing a range of real-time PCR experiments. Background information is included at the start of each chapter to help familiarize the user with the theory of operation and instrument setup.

This guide makes the assumption that the reader has a basic understanding of:

- Laboratory techniques; including assay design and preparation
- Microsoft Windows including terminology, common commands, file saving etc.

The guide is separated into the following main areas:

Chapter	Title	Content
Chapter 1	Safety and installation information	Instrument specifications Warranty and certification Site requirements; PC requirements Installing Quansoft applications software Filter installation
Chapter 2	Introduction to PrimeQ and real-time PCR	Overview of real-time PCR Examples of fluorescent chemistries PrimeQ system overview
Chapter 3	Using Quansoft	Introduction to Quansoft PCR program setup Plate layout setup Analysis setup Starting/running/stopping/editing an experiment
Chapter 4	Data analysis	Introduction/theory of data analysis Quantification Dissociation curve Plus/minus scoring Allelic discrimination End-point analysis (Multi-read) Multiplexing
Chapter 5	Technical information	Maintenance and spare parts Technical support

Chapter 6	Troubleshooting and Glossary	Identifying problems with the instrument and/or the PCR Glossary of terms used in real-time PCR

Declaration of Conformity

Real-Time Thermal Cycler Model, PrimeQ

These products comply with the requirements of the EU Directives listed below:

2004/108/EC **EMC Directive.**
2006/95/EC **Low Voltage Directive (LVD).**

Compliance with the requirements of these Directives is claimed by meeting the following standards:

EN 61326-1:2006 (Electrical Equipment for Measurement, Control and Laboratory use).

EN 61010-1: 2001 (Safety Requirements Electrical Equipment for Measurement, Control and Laboratory use).

EN 61010-2-010: 2003 (Particular Requirements for Laboratory Equipment for Heating of Materials).

CE mark affixed 2012.

Signed: _____ (Mr S. Marriott)

Date: 7/8/12

Authority: Technical Director
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Contents

1 Introduction

Safety and installation information

About this chapter

This chapter provides information on general safety aspects, definitions, advice and instructions for unpacking and installing your instrument. It also gives general information about the instrument and the control software, system requirements, basic procedures, control mechanisms and software installation.

1.1 PrimeQ

PrimeQ - the real-time nucleic acid detection system from Techne - has been designed with the advantage of an open chemistry format that allows the end user full flexibility in the methods and research they wish to pursue. This is complimented by the user-friendly application software, Quansoft, for easy setup of experiments and rapid analysis of data.

1.1.1 PrimeQ features

- The white light source and PMT detector provide an impressive excitation range of 470nm to 650nm and a detection range of 500nm to 710nm.
- Multiplex - multiple wavelengths are detectable per sample using up to four paired excitation and emission filters housed in individual cartridge systems.
- Wide dynamic range up to at least nine orders of magnitude from starting copy number and high sensitivity detecting down to 1.0nM fluorescein and single copy templates, depending upon the assay.

1.1.2 The thermal system

- 96-well low-profile microplate format sealed with optical film.
- Temperature-controlled optical heated lid can be set between 100°C and 115°C (or off). Designed to minimize loss of sample and prevent sample condensation.
- Ramp rates up to 2.2°C/sec.
- Temperature range 4°C to 98°C.
- Block uniformity of less than ±0.25°C.

1.2 Quansoft

Accompanying PrimeQ is Techne's unique, intuitive wizard-based software, Quansoft. By employing a series of user-friendly windows accessible from the home page, Quansoft enables any real-time experiment to be created with ease.

1.2.1 Experiment Editor

By using combinations of plate layouts, thermal cycling programs and analysis parameters the Quansoft Experiment Editor allows the user easy management of experimental protocols.

1.2.2 Plate Layout Editor

Within a matter of seconds a 96-well microplate can be assigned with blanks, controls, standards, user-defined samples or unknown samples, all of which are colour-coded for ease of identification.

1.2.3 Program Editor

Individual cycle steps, stages or temperature ramps can be added quickly and easily together with other parameters to build and display the thermal cycling program.

1.2.4 PrimeQ reports

Comprehensive, completely user-customizable reports can be generated from any of the analysis methods. All result types including graphs can be exported to Microsoft® Word, PowerPoint or other programs currently used for publication purposes.

1.2.5 Data Analysis

Choose the analysis types and parameters from:

- None
- Baseline
- Quantification
- Dissociation curve
- Plus/minus scoring
- Allelic discrimination
- Multi-read (end point)

1.2.5.1 Quantification

A linearity of at least nine orders of magnitude can be achieved, allowing the user to quantify DNA down to single copy template or to achieve absolute quantification of 1.0nM fluorescein in a volume of 20µl.

1.2.5.2 Dissociation analysis

Using the easy-to-program 'ramp' function, PrimeQ will perform a dissociation curve that can be used to determine the temperature at which the DNA strands dissociate. It provides the user with extra confidence in genotyping experiments and in product verification analysis.

1.2.5.3 Plus/minus scoring

This analysis exploits PrimeQ's fluorescence technology to determine with ease and accuracy the presence or absence of a PCR product in any given sample.

1.2.5.4 Allelic discrimination

Users of PrimeQ have the option of this powerful technique capable of detecting single nucleotide differences (SNPs). It can be used to discriminate between genotypes, mutations and polymorphisms within or between samples simply by comparing the fluorescence signal obtained using allele-specific, dye-labelled probes.

1.3 Unpacking

When unpacking the unit, check that the following have been removed from the packing:

- This PrimeQ operator guide
- A CD containing an electronic copy of this guide and Quansoft software
- The PrimeQ unit
- A thermal block
- Filter cartridges in box
- Mains lead(s)
- Guarantee card
- Decontamination certificate
- USB cable

Keep the original packaging in case you ever need to return the unit for service or repair. Techne accepts no responsibility for damage incurred during transportation unless the unit is correctly packed and transported in its original packaging.

The instrument weighs 25kg (55lb). Take care when lifting – this is best done by two people.

1.4 Warning!

Position the unit so that the mains on/off switch is accessible. If a safety problem should be encountered, switch off at the power socket and remove the plug from the supply.

If you are using more than one unit in close proximity to each other, there must be at least 100mm between the units to allow the cooling air to flow correctly.

1.5 Uses of PrimeQ

PrimeQ has many scientific laboratory applications. Aspects of the PCR process are claimed in U.S. Patent Nos. 5,475,610 (claims 160-163 and 167) and 6,703,236 (claims 1-6) or corresponding claims in their non-US counterparts. Use of PrimeQ in such processes is covered by licenses granted to Techne by Applied Biosystems LLC (except for use in human in vitro diagnostics).

1.6 Plates

Techne recommends only the type of low-profile plate described in this Guide using reaction volumes between 15µl and 50µl. Plates must be able to withstand the temperatures you are using without any danger of deforming to the point where they fracture. Only low-profile plates should be used. If standard height PCR plates are used, PrimeQ may be damaged and the warranty will be invalid. Likewise if you put sections of cut-up plate in the block you must balance the plate sections with empty sealed plate sections or previously used plate sections to balance the pressure on the heated lid.

During the final cool-down, a ring of condensation may form on the inside walls of the plate wells, above the liquid level but below the top of the sample block. This is not usually a cause for concern as the condensation does not form during cycling, due to the action of the heated lid.

1.7 Thermal seal

The specification given in this Guide is based on the use of an optical heat seal. Other types of optical seal may be used; however any seal must be able to withstand the temperatures you are using without any danger of deforming to the point where it splits. The user should check the suitability of the seal to be used to ensure there is minimal sample evaporation.

Where applicable, you should use a heat sealer which seals at a temperature of approximately 170ºC.

Place the plate in the heat sealer base plate, cover it with the seal. Seal the plate for 3-4 seconds; rotate the plate 180 degrees; seal the plate again for a further 3-4 seconds.

The plate is now correctly sealed and ready for inserting into PrimeQ.

Always be certain that you use the film in the correct way. If you find that you have used the seal the wrong way up DO NOT PLACE IT IN THE PRIMEQ UNIT as it will damage the heated lid and this will invalidate the warranty.

Clean the heat sealer before you try to seal another plate.

When using the recommended brand of thermal seal, FHSFILM, and find that it has been applied the wrong way, the user is advised to send the heat sealer back to their supplier for repair.

Should the user want to attempt in-house repair, the heat sealer should be left to cool and the heating plate cleaned according to the manufacturer's instructions.

1.8 Contacting us

If you have any questions about PrimeQ, our technical support resources can be accessed in the following ways:

- Internet: Visit our websites at www.techne.com or www.techneusa.com to find out the answers to a range of frequently asked questions.
- Email: Contact PrimeQHelp@bibby-scientific.com or PrimeQHelp@techneusa.com for technical and applications assistance. For servicing enquiries contact service@bibby-scientific.com or service@techneusa.com in the US.
- Phone: For technical support call +44 (0)1785 810433. For servicing call +44 (0)1785 810475 or +1 609 589 2560 in the US.

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1.9 Installing the hardware

To protect the mechanisms within the PrimeQ, the thermal cycling block is packed and transported separately from the instrument (in the main carton).

DO NOT PLUG IN THE POWER CABLE OR TRY TO SWITCH ON UNTIL YOU HAVE INSTALLED THE BLOCK.

See the instructions in sections 1.10.

1.9.1 Site requirements

PrimeQ can operate on a laboratory bench top. The unit is 420mm wide x 518mm deep x 528mm high and requires 100mm side clearance for operation. The unit weighs 25kg (55lb).

The unit should ideally be operated in the temperature range of 18°C to 30°C out of direct sunlight and draughts. Operating humidity range should be RHa up to a maximum of 80% non-condensing.

The unit can be connected to a power supply with voltages from 100 to 230V AC.

The unit must be connected to a PC as described in section 1.14.

1.9.2 Installing the instrument

1.9.2.1 Warning

HIGH TEMPERATURES ARE DANGEROUS: they can cause serious burns to operators and ignite combustible material.

Techne has taken great care in the design of these units to protect operators from hazards, but users should pay attention to the following points:

- USE CARE AND WEAR PROTECTIVE GLOVES TO PROTECT HANDS;
- DO NOT put hot objects on or near combustible objects;
- DO NOT operate the unit close to inflammable liquids or gases;
- DO NOT place any liquid directly in your unit;
- At all times USE COMMON SENSE.

1.9.2.2 Aviso

LAS TEMPERATURAS ELEVADAS SON PELIGROSAS: pueden causarle graves quemaduras y provocar fuego en materiales combustibles.

Techne ha puesto gran cuidado en el diseño de estos aparatos para proteger al usuario de cualquier peligro; aun así se deberá prestar atención a los siguientes puntos:

- EXTREME LAS PRECAUCIONES Y UTILICE GUANTES PARA PROTEGERSE LAS MANOS;
- NO coloque objetos calientes encima o cerca de objetos combustibles;
- NO maneje el aparato cerca de líquidos inflamables o gases;
- NO introduzca ningún líquido directamente en el aparato;
- UTILICE EL SENTIDO COMUN en todo momento.

1.9.2.3 Avertissement

DANGER DE TEMPERATURES ELEVEES : les opérateurs peuvent subir de graves brûlures et les matériaux combustibles risquent de prendre feu.

Techne a apporté un soin tout particulier à la conception de ces appareils de façon à assurer une protection maximale des opérateurs, mais il est recommandé aux utilisateurs de porter une attention spéciale aux points suivants:

- PROCEDER AVEC SOIN ET PORTER DES GANTS POUR SE PROTEGER LES MAINS.
- NE PAS poser d'objets chauds sur ou près de matériaux combustibles.
- NE PAS utiliser l'appareil à proximité de liquides ou de gaz inflammables.
- NE PAS verser de liquide directement dans l'appareil.
- FAIRE TOUJOURS PREUVE DE BON SENS.

1.9.3 Operator safety

All users of Techne equipment must have available the relevant literature needed to ensure their safety.

It is important that only suitably trained personnel operate this equipment in accordance with the instructions contained in this Guide and with general safety standards and procedures. If the equipment is used in a manner not specified by Techne, the protection provided by the equipment to the user may be impaired.

All Techne units have been designed to conform to international safety requirements and are fitted with an over-temperature cut-out.

If a safety problem should be encountered, switch off at the power switch and remove the plug from the supply.

1.9.3.1 Seguridad del usuario

Todos los usuarios de equipos Techne deben disponer de la información necesaria para asegurar su seguridad.

De acuerdo con las instrucciones contenidas en este manual y con las normas y procedimientos generales de seguridad, es muy importante que sólo personal debidamente capacitado opere estos aparatos. De no ser así, la protección que el equipo le proporciona al usuario puede verse reducida.

Todos los equipos Techne han sido diseñados para cumplir con los requisitos internacionales de seguridad y traen incorporados un sistema de desconexión en caso de sobretemperatura. En algunos modelos el sistema de desconexión es variable, lo que le permite elegir la temperatura según sus necesidades. En otros, el sistema de desconexión viene ya ajustado para evitar daños en el equipo.

En caso de que surgiera un problema de seguridad, desconecte el equipo de la red.

1.9.3.2 Sécurité de l'opérateur

Tous les utilisateurs de produits Techne doivent avoir pris connaissance des manuels et instructions nécessaires à la garantie de leur sécurité.

Important : cet appareil doit impérativement être manipulé par un personnel qualifié et utilisé selon les instructions données dans ce document, en accord avec les normes et procédures de sécurité générales. Dans le cas où cet appareil ne serait pas utilisé selon les consignes précisées par Techne, la protection pour l'utilisateur ne serait alors plus garantie.

Tous les appareils Techne sont conçus pour répondre aux normes de sécurité internationales et sont dotés d'un coupe-circuit en cas d'excès de température. Sur certains modèles, ce coupe-circuit est réglable pour s'adapter à l'application désirée. Sur d'autres modèles, il est pré-réglée en usine pour assurer la protection de l'appareil.

Dans le cas d'un problème de sécurité, coupez l'alimentation électrique au niveau de la prise murale et enlevez la prise connectée à l'appareil.

1.9.4 Installation

1. All Techne units are supplied with a mains cable. This is a plug-in lead type cable.
2. Before connecting the power supply, check the voltage against the rating plate. Connect the mains cable to a suitable supply according to the table below.

   Note that the unit must be earthed to ensure proper electrical safety.

   100V to 230V

   Live Black

   Neutral White

   Earth Green

The fused plug supplied with the mains cable is fitted with a 10 amp fuse to protect the instrument and the operator.

The rating plate is on the rear of the unit.

Note: the unit can work in the range 100V to 230V.

3. Plug the mains cable into the socket on the rear of the unit.
4. Place the unit on a suitable bench or flat workspace, or in a fume cupboard if required, ensuring that the air inlet vents on the underside are free from obstruction.
5. Symbols on or near the power switch of the unit have the following meanings:

   O: power switch Off

   I: power switch On

1.9.4.1 Instalación

1. Todos los aparatos Techne se suministran con un cable de alimentación. Puede ser fijo o independiente del aparato.
2. Antes de conectarlo, compruebe que el voltaje corresponde al de la placa indicadora. Conecte el cable de alimentación a un enchufe adecuado según la tabla expuesta a continuación.

   El equipo debe estar conectado a tierra para garantizar la seguridad eléctrica.

   100V - 230V

Linea	Negro
Neutro	Blanco
Tierra	Verde

   El fusible (10A) una vez instalado protege tanto al equipo como al usuario.

   Los equipos funcionan a 100V y 230V.

   La placa indicadora está situada en la parte posterior del equipo.
3. Conecte el cable a la toma de tensión en la parte posterior del equipo.
4. Sitúe el aparato en un lugar apropiado tal como una superficie de trabajo plana, o si fuera necesario incluso en una campana con extractor de humos, asegurándose de que las entradas de aire en la parte inferior no queden obstruidas.
5. Los símbolos que se encuentran en o cerca del interruptor de alimentación tienen los siguientes significados:

   O: Interruptor principal apagado

   I: Interruptor principal encendido

1.9.4.2 Installation

1. Tous les appareils Techne sont livrés avec un câble d'alimentation qui peut être intégré à l'appareil ou à raccorder.
2. Avant de brancher l'appareil, vérifiez la tension requise indiquée sur la plaque d'identification. Raccordez le câble électrique à la prise appropriée en vous reportant au tableau ci-dessous.

   Il est important que l'appareil soit relié à la terre pour assurer la protection électrique requise.

   100V - 230 V

Phase	Noir
Neutre	Blanc
Terre	Vert

Le fusible (10A) à l'intérieur de l'appareil est destiné à assurer la protection de l'appareil et de l'opérateur.

Les appareils fonctionner sur 100V et 230V

La plaque d'identification se trouve à l'arrière de l'appareil.

3. Raccordez le câble d'alimentation à la prise située à l'arrière de l'appareil.
4. Placez l'appareil sur un plan de travail ou surface plane, ou le cas échéant, dans une hotte d'aspiration, en s'assurant que les trous d'aération situés sous l'appareil ne sont pas obstrués.
5. Les symboles situés sur ou à côté de l'interrupteur de l'appareil ont la signification suivante:

   O: Arrêt

   I: Marche

1.10 Before switching on

1.10.1 PrimeQ front view

1.10.2 Insert the block

Push firmly on the front of the drawer to open it and insert the block.

- Slide the quick release handles in the direction shown on the top to the unlock position.
- Lift the block assembly into the unit. Never lift or carry the block by one side, always use both sides (or support the block from underneath).
- Ensure the block is fully engaged by pushing all four corners of the block down into the carrier.
- Slide the quick release handles in the opposite direction to lock the block assembly in position.
- Push the drawer back into the unit.

1.10.3 Fuses

Ensure that the correct fuses are fitted for your voltage supply.

Fuses should only be changed by a competent person with training if necessary.

For voltages from 110 V to 130 V, use T10A fuses only.

1.10.4 Connections on the back of the unit

There are two cable connections: the mains power switch and the USB socket located on the rear of the PrimeQ.

A. USB computer connection. Plug the USB lead into the socket and connect the other end to your computer. The lead must be less than 2 meters in length (the lead supplied with the instrument is correct).

B. Power switch.

C. Fuses

D. Power connection. Ensure that the switch is in the off, O, position. Plug the mains cable into the power socket.

When PrimeQ is sited in the correct position and all the connections have been made, turn the switch to the on (I) position on the PrimeQ.

1.11 Technical Specification

Thermal cycler

Block Format:	96 x 0.2ml well low-profile micro tube plate
Block Specification:	8 x Peltier block employing quad circuit technology to enhance performance
Block Uniformity at 50ºC:	< ± 0.25ºC
Maximum Heating Ramp Rate:	2.2ºC/sec
Temperature Range:	4ºC to 98ºC
Sample Volume:	15µl to 50µl
Heated Lid:	Adjustable between 100ºC and 115ºC or off

Optical detection system

Excitation Source:	White LED
Detector:	Photon counting photo multiplier tube (PMT)
Multiplex Dye Detection:	Up to four dyes per reaction tube
User Selected Filters:	Maximum of four paired excitation/emission filter cartridges suitable for use with currently available dyes
Fluorescence Excitation Range:	470nm to 650nm
Fluorescence Detection Range:	500nm to 710nm
Dynamic Range:	At least nine orders of magnitude from starting copy number.
Sensitivity:	1.0nM fluorescein in a 20µl sample Single initial template copy detection*

Dimensions

Weight:	25kg
Size and Footprint:	420mm x 528mm x 518mm (W x H x D)
Power Supply:	100V to 230V, 50/60Hz
Input Power:	715W
IP code:	IP30

* *Assay dependent*

Computer requirements (Not Supplied)

The following are the recommended minimum PC specifications required for running PrimeQ:

CPU:	Core 2 Duo or equivalent
Memory:	2Gb DDR RAM as a minimum
Storage:	40Gb DMA hard drive
Display:	1024 x 768 resolution minimum, 17 inch digital monitor recommended.
Drive:	DVD/CD-RW drive
Operating System:	Microsoft® Windows® XP Professional SP3 or later, Windows® Vista, Windows® 7
Connections:	USB
Also useful:	
Sound:	Built-in sound, video and LAN facilities
Internet:	Ethernet connection
Software:	Internet Explorer, Microsoft® Office

PC must be operated in 'always on' mode, no hibernation or sleep mode allowed.

1.12 Working conditions

PrimeQ is designed to work safely under the following conditions:

Ambient temperature range:	5°C to 40°C
Humidity:	Up to 80% relative humidity, non-condensing.

Notes:

The control specifications are quoted at an ambient temperature of 20°C.

The specification may deteriorate outside an ambient temperature range of 18°C to 30°C.

Warning:

The unit may be damaged if used in an ambient temperature above 40°C.

1.13 Guarantee

Please read this important GUARANTEE information.

Notwithstanding the description and specification(s) of the units contained in the Operator Guide, Techne hereby reserves the right to make such changes as it sees fit to the units or to any component of the units.

This Guide has been prepared solely for the convenience of Techne customers and nothing in this Manual shall be taken as a warranty, condition or representation concerning the description, merchantability, fitness for purpose or otherwise of the units or components.

The PrimeQ unit is guaranteed for a period of 2 years from the date of purchase.

Within these periods we undertake to supply replacements free of charge for parts which may on examination prove to be defective, provided that the defect is not the result of misuse, accident or negligence.

On all correspondence, please quote the Serial Number in full and/or the Sales Order Number.

Any instrument requiring service under this guarantee should be taken to the supplier through whom it was purchased, or, in the case of difficulty, it should be carefully packed in its original packing and consigned, carriage paid, to us. Techne takes no responsibility for returned goods damaged in transit.

Returned goods will not be processed without a Returns Authorization Number.

Call the Service Department on +44 (0)1785 810475 or +1 609 589 2560 in the US for a Returns Authorization Number.

Please write the Returns Authorization Number on the outside of any packing and ensure that a copy of a Decontamination Certificate is visible.

Please register online or complete and return the Registration Card to the address on the card. Returning the card or registering will help us to contact you with new information about Techne products and product up-dates thus improving our service to you.

The Guarantee will be rendered invalid if any of the small labels shown opposite are broken or missing when the unit arrives at the supplier's under any claim for warranty repairs.

1.14 Installing the application software

Always set your PC or laptop from which you are running PrimeQ so that standby is switched OFF. This can easily be achieved through the control panel or:

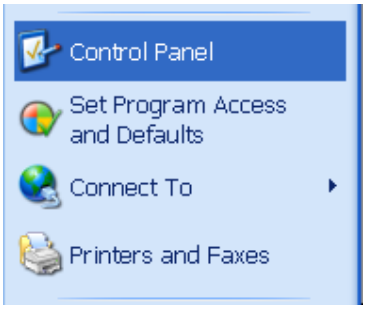
- Right click on the desktop and select **Properties**.
- Select the **Screen Saver** tab and click on the **Power** button.
- Activate the power scheme entitled **Always On** from the drop down menu and click **Apply**. You may need to restart your computer.

1.14.1 Set the decimal symbol to‘.’

Before installing this software please ensure that the decimal symbol on the computer is set to ‘.’. If it is not then follow these instructions:

- Click on the **Start** button:

- Go to **Control Panel**:



- Select **Regional and Language Options**:

- Ensure the decimal in the number section is set to a ‘.’

1.14.2 Loading Quansoft onto the PC

To install the Quansoft software onto the PC, follow these five basic steps:

1. Turn on the PC and log on as a user having administrator rights (to be able to install software).
2. Insert the Quansoft CD into the CD-ROM drive and wait for the auto-run function to automatically start the setup (should auto-run fail to start, then the CD must be opened from Explorer and the setup program (Setup.exe) initiated manually by double-clicking).
3. Click on the **Install** button and wait for the installation to complete. Quansoft files will automatically be installed to C:\Programs\Techne\Quansoft. If you wish to change the destination click the **Browse** button and choose accordingly. You will also be given the option of creating a shortcut icon from the desktop for easy access.
4. Remove the disk from the drive.
5. Restart the computer, log on and then launch Quansoft from the **Programs** menu or via the shortcut icon on the desktop.

The software can be easily uninstalled from the computer: simply access the PC **Control panel**, click on **Add/remove programs** and then select **Quansoft**.

If upgrading from one release of Quansoft to another, then the previous release should first be uninstalled as described.

Note: the Release number should not be confused with the version numbers which relate to each of the Quansoft editors.

1.14.3 Registering the software

A registration welcome screen will appear when launching Quansoft for the first time. By registering your software with Techne, you will be eligible for technical support and software updates posted on the Techne website. However, the software can still be used for a 28-day trial period without registration – simply click **Register Later** and the prompt screen will appear next time the software is opened. On pressing **Register Now**, a form will be displayed requiring the following fields to be completed: name, organization, unlock code and the instrument serial number, should one be connected.

The unlock code can be obtained in one of three ways:

1. **Internet:** Clicking on www.techneusa.com/PrimeQregister in the registration panel, you will be taken directly to the PrimeQ registration page.
    a. Enter your details as listed on the screen.

b. An unlock code will be e-mailed to you within one working day.

Ensure you are online before attempting to do this.

2. **E-mail:** PrimeQhelp@bibby-scientific.com or PrimeQHelp@techneusa.com.

   Send an email complete with details of your name, company or institution, mailing address, e-mail address, telephone number, instrument and block serial number (if you have a PrimeQ) together with the unique registration number.

   Exit the registration procedure by pressing 'cancel' and return when the unlock code has been returned by email.

3. **Phone:** Provide us with your details and registration number by telephoning: +44 (0)1785 810433.

1.14.4 Software upgrades

Development of the PrimeQ control and analysis software is an ongoing process. To check your version number, click 'Help/About Quansoft' and a splash screen displaying the current details will appear.

Check the Techne websites at www.techne.com or www.techneusa.com for the most current software updates. These updates can be downloaded free of charge for registered users.

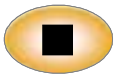
1.15 Using the LCD control panel

1.15.1 Function keys

PrimeQ is controlled primarily from the PC using the application software, although some basic functions can be performed via the LCD screen located on the front of the instrument.

The display indicates the instrument name, current status and basic information about any experiment currently running such as its progression. The keypad has three function buttons:

Start/Pause: Starts the run or pauses if already running.

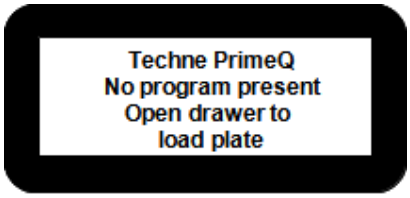


Stop: Stops the current run. The screen will ask for confirmation of the stop command. This button can be deactivated from Quansoft to guard against accidental stoppage.

Info: Information about the program currently running on the unit can be obtained at any time during the run.

1.15.2 Power up screen

The screen is a 20 character, four row LCD. There is a four button keypad.



1.15.3 Beeper

A beeper is used typically to:

- Inform the user of any error made when interacting with the instrument (such as an invalid key press).
- Indicate a change of state of the instrument during a run (such as run completed).

1.15.4 Before you start

Check that:

1. All cables are connected between the instrument and PC.
2. All power cables are plugged in and comply with safety standards.
3. The Quansoft software has been installed on the PC.

1.15.5 Start-up procedure

1. Turn on the power to PrimeQ by pressing the switch at the back of the instrument. A welcome beep can be heard.
2. Turn on the PC and wait for Windows to boot up.
3. Log in under your user name.
4. Double-click the Quansoft icon on the desktop to launch the software.

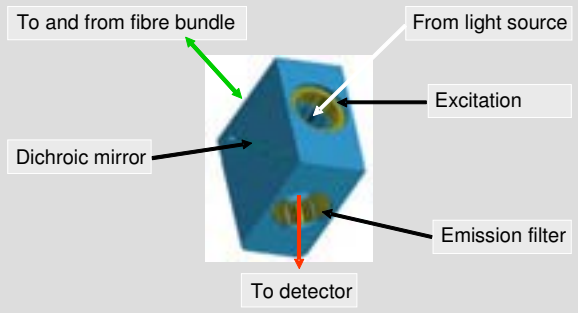
PrimeQ is now ready for use.

1.16 Installing the filter cartridges

1.16.1 The role of the filter cartridge

The role of the filter cartridge is to control the excitation and emission wavelengths of light using filters and a dichroic mirror.

- The excitation filter transmits only the wavelength of the light source that excites the fluorophore.
- The emission filter transmits only the wavelength of the light produced by the fluorescence of the sample.



1. White light from the LED light source enters the cartridge and is separated by the excitation filter.
2. Light of the desired wavelength is then turned 90° by a dichroic mirror and directed to the wells via the fibre optic bundle.
3. Emitted light produced by the excited fluorophores is transmitted via the fibre optic bundle, straight through the dichroic mirror and filtered by the emission filter such that only light at the correct emission wavelength is allowed through.
4. Emitted light is passed on to the photo-multiplier tube (PMT) for detection.

The filters provided with the system are high-quality components with special anti-reflective coatings. As with all filters, they will degrade over time, especially in climates with higher humidity levels. This will ultimately lead to degradation in the instrument performance such that the purchase of replacement filter cartridges is required (see section 5.6). Filters should last at least three years, possibly as long as five depending on the frequency of use and conditions.

1.16.2 Filter cartridge care instructions

- The filter cartridges must be handled with special care.
- Only lift them from the foam in the box by the sides of the cartridges.
- Avoid touching the filters.

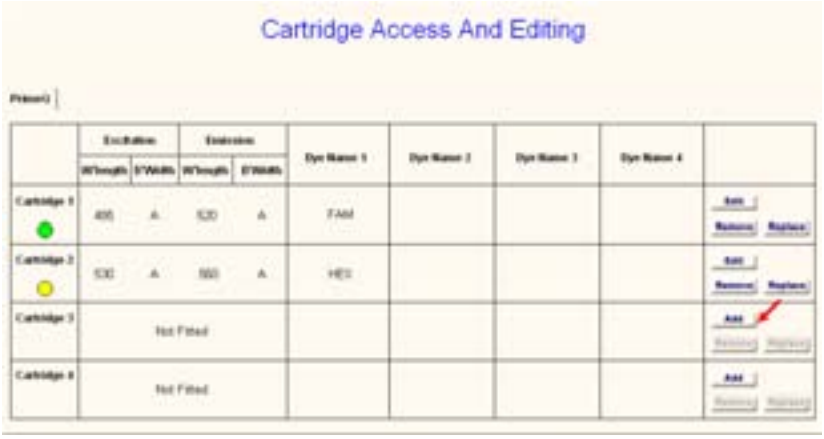
Loose particles should be removed with a bulb puffer or filtered, pressurized air cleaner. If necessary, gently wipe the surface using 100% alcohol and optical wipes; use a clean optical wipe each cleaning motion.

1.16.3 Filter cartridge installation

The filter cartridges are positioned in a carousel with each cartridge having an identification label visible from the access door. Installing filter cartridges into the carousel is a relatively simple procedure that can be carried out by the user.

- In Quansoft, access the filter settings screen by clicking on the **Cartridge** icon located on the navigation bar of the Home Page (found under the **Maintenance** tab).

This will bring up the **Cartridge Access and Editing** screen. An instrument must be connected for this screen to be displayed.



- **Cartridge position:** Colour-coded for ease of identification.
- **Add/Remove/Edit/Replace:** Launches a wizard which leads the user through the requested procedure.

- **Excitation/emission wavelengths:** As defined in the **Add Cartridge** settings box.
- **Dye name:** Each cartridge can be assigned up to four different dye names of the user's choice (e.g. FAM, fluorescein, SYBR® Green etc.).

1.16.4 Adding a filter cartridge

- In the Access and Editing screen of the Filter Wizard, click the **Add** button next to the appropriate filter. The **Add Cartridge** screen will appear.

- Set the wavelengths and bandwidths for the excitation and emission filters.

This information can be found on the side of the filter cartridge.

These filter details become the filter ID such that the fluorophores are viewed or chosen by the dye name e.g. FAM or Cy5. The input table can hold details for the four filter positions of the carousel.

- Click **Next**.

A screen appears prompting the user to perform a five-step procedure.

1. Lift the top lid of the PrimeQ.
2. Lift the cartridge/carousel cover.
3. Insert the cartridge and ensure that the magnets engage to hold it in place; you will hear a "click" as the magnets engage. It is important that the cartridges are fitted correctly.
4. Close both lids.
5. Once the cartridges have been installed in the unit, it is the user's responsibility to assign filter descriptions so that the application software knows what type of filter is located in which position of the carousel.

1.16.5 Assigning filter descriptions in Quansoft

Once the cartridge has been fitted into the cartridge carousel, the system will check for the presence of a new cartridge and then ask the user for the **Dye Names**. Up to four names can be assigned to any given filter cartridge and the colour coding can be changed by clicking on the **Colour** icon. The colour chosen here will be the colour of the dot representing the read points on the thermal profile graph.

The assigned cartridge will now be available for use in the **Program Editor** (see section 3.4.3). It is represented by the icon colour chosen during installation, although the colour can be changed by double-clicking the icon in the read settings box. The user can choose to select a cartridge by name or by wavelength. Simply click the **Select by Name/Wavelength** button to select which filter to use.

1.16.6 Editing filter descriptions

Clicking the **Edit** button in the Cartridge Access & Editing screen brings up the settings boxes so that it is possible to change the wavelength, bandwidth, name or icon colour for the filter. Perform the steps in the same way as when adding a cartridge.

1.16.7 Removing a filter cartridge

If a cartridge is placed into the filter carousel without being installed via the software, a message will warn the user of the presence of an **illegal cartridge** and the cartridge will be unavailable for use. To remove this or any other cartridge, use the following procedure:

- In the Access and Editing screen of the Filter Wizard, click **Remove** next to the appropriate filter.
- Click **Yes** to confirm the procedure and the **Remove Cartridge** screen will appear.
- Follow the procedure as shown and click **Finish** to complete.

A message will appear informing the user to **Please wait – checking cartridge status**.

If the removal was performed correctly, the Cartridge Access and Editing table will be updated to display “Not Fitted” next to the slot from which the cartridge was removed.

The table below gives details of the PrimeQ filter cartridges.

Filter cartridge	Excitation wavelength (nm)	Emission wavelength (nm)	Suitable dyes
FC01	460	500	FAM multiplex
FC02	485	520	FAM/SYBR®
FC03	530	560	HEX/TET/JOE/VIC/Yakima Yellow
FC04	580	615	ROX
FC05	640	685	Cy5/Quasar® 670

1.16.8 Points to remember

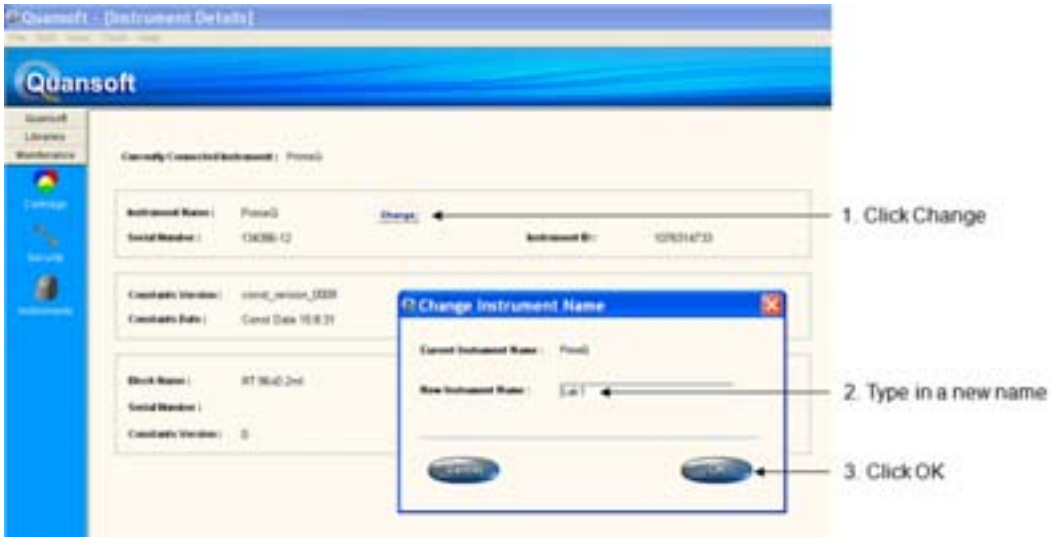
- Filter descriptions are stored in the instrument memory and do not need to be re-entered if the connected instrument is changed to a different PC. Filter descriptions are uploaded from the instrument when Quansoft is opened.
- Changing the filter cartridge between experiments can only be carried out via the software – it cannot be performed manually.
- It is critically important that the correct excitation and emission filters are in the assigned position of the carousel.
- During multiplexing you must choose two fluorophores with different excitation and emission wavelengths and measure the fluorescence with different filter cartridges.
- Avoid combinations of fluorophores with overlapping spectra.
- It is possible for some fluorophores to appear more than once on the dye list because more than one filter pair could be used for its measurement. In this case, select the correct filter by its wavelength properties.

1.17 Changing the instrument name

All PrimeQ units are shipped with the name “PrimeQ” stored in the instrument’s software. This can be changed to a more appropriate name as required.

- To change the instrument name, click on the **Maintenance** icon on the navigation bar. Then click on the **Instruments** icon. The default supervisor password is **techne** – we suggest you change this as soon as possible by clicking on the **Security** icon. Please ensure that you keep a record of the password, as without it you will not be able to access these functions.

- On the instrument screen, click on **Change,** type in the new name and then click **OK**.



Once the name has been changed you need to switch off the instrument before the new name can appear on the LCD screen and take effect.

The maintenance screen also gives instrument-specific details including serial number, unique ID and block cycle count. It also shows block constant (calibration) settings.

1.18 LED power settings

The LED light source has variable power settings. This prevents PMT blinding and brings fluorescence into its linear range for certain applications. The role of this option is to cut down excess light from the LED source in high fluorescence applications. The three power options are: Low (50mA), Medium (100mA), and High (200mA).

The power level is specified by the user when programming the PCR in the application software (at the same time as specifying the fluorophore filter); the default setting is **Medium**. The three set power levels are not user-adjustable.

1.19 Inserting a plate

- To insert a plate, push the orange panel to release the latch, and open the drawer by sliding it towards you.
- To close the drawer push it home until the latch clicks and the drawer stays shut.

1.19.1 Inserting a previously run plate

Users should note that when the thermal cycler plates are heated, the plastic may tend to distort slightly. This means that if the plate is removed from the block then either returned or transferred to another block, the fit can be poor. If returning or transferring a plate is unavoidable then the following procedure is recommended to improve the fit:

- If a heat seal has been used, place the plate in the heat-sealer and seal the plate for 3-4 seconds; rotate the plate 180 degrees; seal the plate for 3-4 seconds.
- Insert the plate in the block and close the drawer.

1.19.2 Running a plate

Perform the loading procedure on the prepared plate following the instruction given when the experimental run is started in the Quansoft operating software.

1.20 After use

When the samples have finished thermal cycling, remember that parts of the unit, such as the tubes, blocks and associated accessories, may be very hot. Take the precautions listed earlier.

Después de su uso

Cuando haya finalizado el calentamiento de muestras, recuerde que las piezas del equipo, tales como tubos, bloques y demás accesorios, pueden estar muy calientes. Tome las precauciones mencionadas anteriormente.

Après utilisation

Lorsque vous avez fini de chauffer les échantillons, n'oubliez pas que certaines parties de l'appareil - les éprouvettes, leurs supports et autres accessoires - risquent d'être très chaudes. Il est donc recommandé de toujours prendre les précautions citées plus haut.

2 Getting started

Introduction to PrimeQ and real-time PCR

About this chapter

This chapter gives a description of PrimeQ together with a basic introduction to real-time PCR. It also provides examples of some of the fluorescent chemistries that can be used with PrimeQ.

2.1 Introduction to PrimeQ

2.1.1 Principle of a real-time PCR instrument

A real-time polymerase chain reaction (PCR) system performs three main functions:

- Cycles the PCR reagents through the specified temperature profile using a specially designed thermal block.
- Excites the fluorescent dye at the appropriate wavelength and at the appropriate point(s) in the PCR program.
- Detects the emitted fluorescence in each well during the specified read point - this information is then fed back to the application software for analysis.

2.1.2 Principle of PrimeQ

PrimeQ is a real-time PCR system that combines a thermal cycler, solid state white light excitation source and a highly sensitive fluorescence detection system all within the convenience of a standard 96-well format. Combined with Quansoft, the system's powerful analysis application software, real-time quantification and analysis is made fast, easy and accurate. PrimeQ has been designed with the advantage of an open chemistry format that gives the user full flexibility in the methods and research they wish to pursue. The system is able to detect as little as 1.0nM fluorescein and achieve a dynamic range of at least nine orders of magnitude†.

In line with the real-time principle, PrimeQ measures the amplification of DNA via a proportional increase in fluorescence which is fed back to the application software. This allows the software to calculate the starting DNA concentration by comparison to a set of standards. The system is capable of measuring up to four different fluorophores per well in real time, thus opening up the possibility of multiplex experiments, where multiple dyes are conveniently used within the same reaction.

The time taken for PrimeQ to read a plate can be adjusted by varying the integration time. Read times can be programmed from 250ms/well integration time (30 seconds/ 96-well plate) down to just 50ms/well (10 seconds/plate) for brighter fluorophores. The default integration time is 150ms/well, which gives a read time of around 20 seconds for a 96 well plate.

2.1.3 Applications of PrimeQ

1. **Quantitative real-time PCR:** Monitoring the accumulation of a PCR product as the run progresses by detecting the increase in fluorescence. Data collection in the early exponential phase of the PCR allows the Quansoft software to accurately calculate initial template quantities.
2. **Qualitative (end-point) PCR:** At the end of a run, the instrument is programmed to read the plate a user-defined number of times for as many fluorophores as present in the samples. The result indicates positive or negative amplification and the data can be analysed in a number of ways to give a qualitative result.
3. **Dissociation point determination:** Samples are slowly heated from the annealing temperature (typically 50 to 65°C) up to the denaturation temperature (~95°C) in small steps. Fluorescence is measured at each step to determine the amount of dye bound to the dsDNA. The point at which the two strands separate is associated with a decrease in the fluorescence signal as the dye can no longer intercalate with the two strands. It is therefore possible to determine the temperature at which 50% of the double-stranded DNA is dissociated (Tm). Since the dissociation temperature of a DNA product is characteristic of the GC content, length and sequence, the Tm can be a useful tool in product identification.

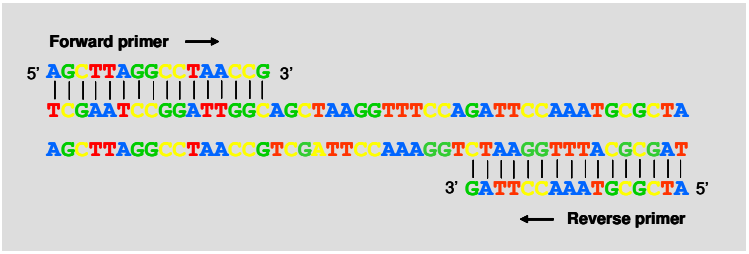
† The dynamic range is assay dependent.

2.2 Introduction to Real-time PCR

2.2.1 PCR

PCR is a powerful biochemical technique that has revolutionised biological research by allowing minute amounts of DNA to be amplified millions of times in just a few hours. PCR allows the selective amplification of a 'target' region of DNA lying between two specific DNA sequences (primers). The DNA sequence lying between these primers does not need to be known, therefore PCR allows researchers to amplify target DNA with relative ease and reproducibility.

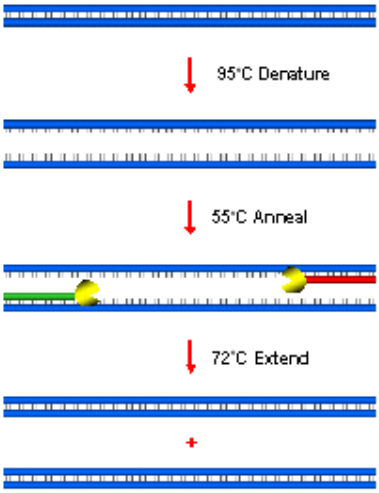
The technique exploits the 5' to 3' polymerase activity of the enzyme *Taq* DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus*. Once the primer binds to the complementary region of the single-stranded target, the enzyme will catalyse the extension of DNA to produce a complimentary second strand.



The primers anneal to complementary regions on the template DNA.

The classical PCR protocol consists of three temperature steps:

1. **Denaturation (at 95°C):** In its normal state, DNA consists of two strands made up of complementary bases. These strands need to be separated before the PCR can progress. The first temperature step is therefore designed to dissociate, or denature, these two strands.
2. **Annealing (typically between 55°C and 65°C):** This temperature step allows annealing of the primers to complementary sequences on the template DNA. The temperature will vary according to the primer characteristics such as GC content, length and sequence.
3. **Extension (72°C):** When the primers have annealed to the complementary single-stranded DNA, the enzyme *Taq* DNA polymerase extends the DNA using its 5' to 3' polymerase activity. The optimal temperature for this enzyme is 72°C.



This results in the production of two new copies of the target DNA which, assuming optimal conditions, can be amplified exponentially by repeating steps 1 to 3.

2.2.2 Qualitative vs. real-time PCR

PCR quickly became an indispensable tool for scientists wanting to amplify and characterize genetic material. However it has one major limitation in that the results are qualitative i.e. it can determine if a target is present but not the amount. The traditional approach to quantification was to compare known sample concentrations of starting DNA with unknown samples cycled at a range of concentrations and cycle numbers. The problems associated with this 'semi-quantitative' approach are many, including the expense of multiple PCR runs, the increased risk of contamination through the need for downstream processing of samples and the fact that end-point measurements have a tendency to vary between replicates. As such, the very accuracy of the post-run method of measurement is put into question. However, real-time PCR or quantitative

PCR (qPCR) extends the usefulness of the technology by permitting the reliable determination of starting DNA template.

2.2.3 Real-time PCR on PrimeQ

Real-time PCR overcomes the limitations associated with the traditional methods by using fluorescence labelling in conjunction with specialized amplification and detection systems to quantify the amount of product being amplified during the PCR process. The fact that downstream processing is eliminated saves both time and money while reducing the risk of contamination. Further savings are to be found with the need for reduced replicates and that multiple reactions can be combined within the same tube using different reporter dyes. However, the most notable advantage over conventional approaches has to be its superiority in terms of accuracy and sensitivity. As a result, this method has become a widely useful tool for the quantification of messenger RNA or DNA levels in a wide range of biological samples.

PrimeQ provides a flexible approach to experiment setup by supporting real-time experiments for monitoring the accumulation of PCR products during thermal cycling as well as end-point assays.

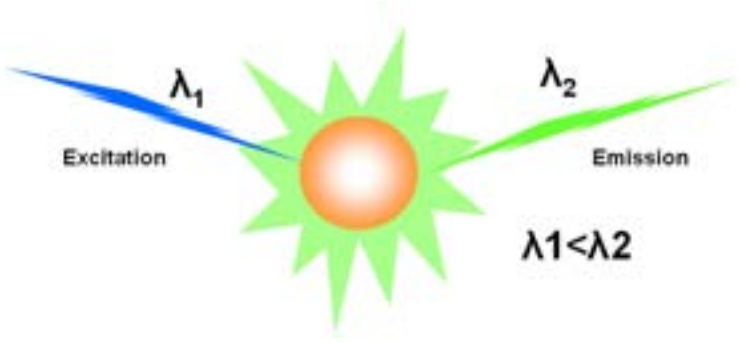
- **Quantitative PCR:** The advantage of this approach is that amplification of the accumulated product is measured in the early exponential phase of the reaction at a point when the amplified product can accurately reflect the starting DNA or RNA levels. By the end of the experiment, reagents could be limiting which may mean that small differences in the PCR performance are magnified. Additional benefits of this approach are its high sensitivity and wide dynamic range. PrimeQ can measure to a sensitivity level of 1.0nM fluorescein and with a dynamic range of at least nine orders of magnitude.
- **End-point:** This approach detects the presence or absence of an amplified product, providing a qualitative positive or negative result. This approach can bring added flexibility to the laboratory, allowing samples that have been run in a different thermal cycler to be quickly screened for the presence of a PCR product by using PrimeQ as a plate reader.

In both approaches, the amount of product present in each reaction tube is visualized using fluorescent reporter dyes.

2.3 Overview of fluorescent chemistries

An extremely sensitive system is required for the accurate visualization of small amounts of PCR products. The conventional approach has been to use radioactivity, which although offering good sensitivity, has obvious disadvantages in terms of safety and environmental risk. Fluorescence has now taken over as the chemistry of choice due to its sensitivity, safety and the excellent flexibility it lends to experimental setup.

Fluorescence is a molecular phenomenon in which a substance absorbs energy in the form of light, causing it to first become **excited** and then to **emit** part of this absorbed energy as light of another colour. This light will be of a lower energy and therefore a longer wavelength (λ) of that absorbed.



Schematic representation of fluorescence.

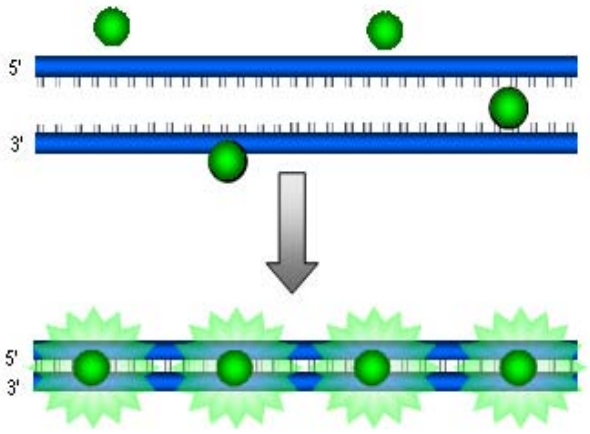
When considering which fluorescent chemistry to use on PrimeQ, there are two main options: fluorescence-labelled probes that bind specifically to the target of interest or intercalating dyes that bind between the two strands of double-stranded DNA. The fluorescence signal seen when the

dye is excited at the appropriate wavelength should be proportional to the quantity of target DNA present. For each type of fluorescent chemistry, the fluorophore will **emit** a fluorescent light that is characteristic of the fluorophore used. A range of spectrally distinct fluorophores are commercially available, introducing the possibility of quantifying multiple targets with different probes in the same reaction well, otherwise known as **multiplexing**.

2.3.1 Intercalating dyes

Intercalating dyes bind to the minor groove of double-stranded DNA (dsDNA) producing up to a thousand-fold increase in fluorescence. Common examples include SYBR® Green I, EvaGreen® and BRYT™Green. As these dyes bind all double-stranded PCR products, this is a universal chemistry with the major advantage of not requiring the use of a fluorescently labelled probe. As such, it is a relatively inexpensive approach and one that is ideal as a screening tool prior to probe manufacture. However, these advantages bring their own disadvantages in that the ability to bind **all** dsDNA means that both specific and non-specific PCR products, including primer-dimers, will be reported.

The signal attributable to each PCR product can however be determined by dissociation curve analysis, a technique that distinguishes PCR products on the basis of melting temperature (Tm). PCR products of different lengths with different GC contents melt or dissociate at different specific temperatures. Dissociation curve analysis thus allows the user, via the specific Tm, to establish that the correct piece of DNA has been amplified. As only a single dye is used to detect all the products, the chemistry is not open to multiplexing i.e. detection of multiple products.



Mode of action of SYBR® Green I:

When the DNA strands are dissociated, the dye does not bind or fluoresce.

SYBR® Green I binds non-specifically to dsDNA. When excited, the emitted fluorescence is relative to the amount of dsDNA present.

The fluorescence will drop when the strands start to dissociate. The temperature at which 50% of the DNA is single stranded is the Tm. Since the Tm is influenced by GC content, length and sequence, it will be indicative of the identity of a PCR product.

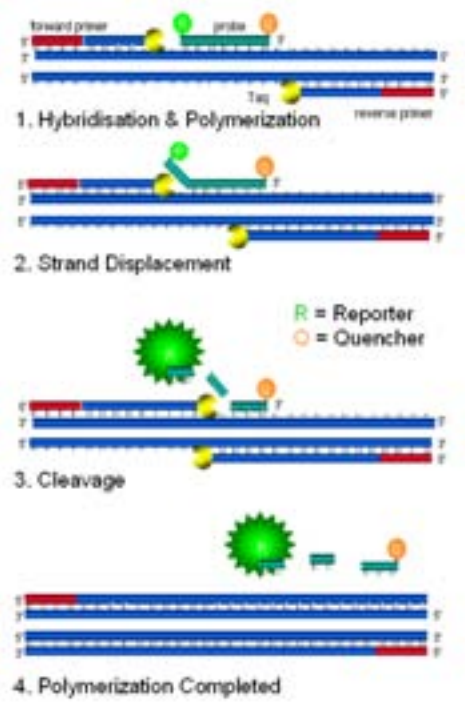
2.3.2 Fluorescent labelled probes

This approach is used when the most accurate quantification of PCR products is required or for when there is more than one target in the reaction tube. Using dye-labelled oligonucleotides that contain a region complimentary to the target sequence to be amplified, provides advantages in terms of high specificity and low background. At the same time, the signal is also ensured to be proportional to the amplified product and not the mass or the length. This chemistry is also multiplex-compatible such that different 'coloured' dyes can be used to report different targets in the same reaction well. Common examples of fluorescent probe chemistries are detailed below.

2.3.2.1 Hydrolysis probes

This chemistry exploits the 5' nuclease activity of *Taq* DNA polymerase to cleave a probe during PCR. The probes are designed as oligonucleotides that are complementary to a region of the target located between the upstream and downstream primer binding sites. The probe contains a fluorophore at the 5' end and a quencher at the 3' end; the close proximity of which means that fluorescence is quenched prior to amplification due to the action of the quencher on the fluorophore. The quencher absorbs the light emitted by the fluorophore with itself emitting either no

fluorescence signal or fluorescence at a longer wavelength which is not detected. The 5' fluorophore is often called the reporter.



Mode of action of hydrolysis probes:

During the PCR, as the DNA polymerase extends the upstream (forward) primer, it encounters the bound probe.

The 5' to 3' exonuclease activity of the polymerase cleaves the probe, releasing the fluorophore into solution, where it is able to fluoresce.

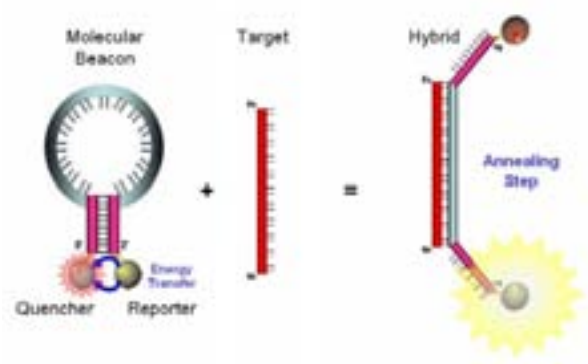
The probe is blocked at the 3' end to prevent extension by the polymerase.

Each cycle of the PCR releases more fluorophore such that the amount of fluorescence in any given cycle should be proportional to the amount of specific product present at any given time.

A particular advantage of the hydrolysis probe technology, aside of the specificity and sensitivity afforded by all the fluorescent probe chemistries, is that the signal accumulation is irreversible. Once a probe is cleaved, the quencher is permanently separated from dye and this is reflected in the signal accordingly.

2.3.3 Molecular Beacons

As with the hydrolysis probe technology, molecular beacons use fluorophore/quencher pairs in their mode of action. When free in solution, molecular beacons assume a hairpin structure that brings the end-bound fluorophore and quencher into close proximity thereby quenching the fluorescent signal. The molecular beacon binds to the amplicon produced during the PCR at a specific temperature when the beacon-target duplex is thermodynamically more stable than the hairpin structure. Binding of the beacon to its target disrupts the hairpin, resulting in spatial separation of the fluorophore from the quencher and allowing it to fluoresce. This increase in fluorescence is reversible as the beacon will dissociate at a higher temperature and close back to a hairpin. The transition from hairpin to bound form is repeated each cycle.



Mode of action of molecular beacons:

When free in solution, molecular beacons assume a hairpin-structure.

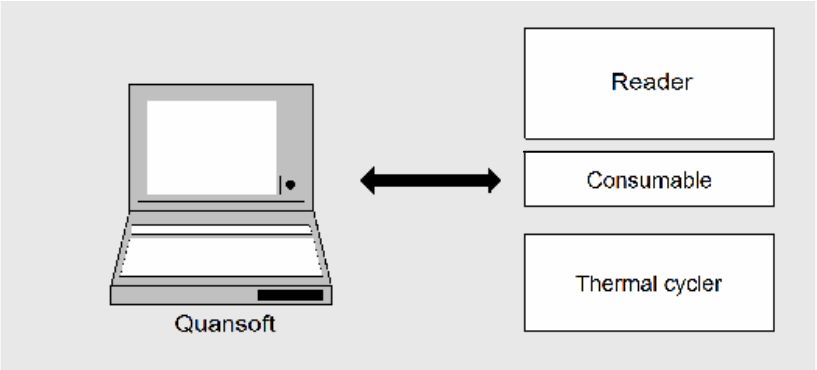
Binding of the beacon to its target disrupts the hairpin, resulting in spatial separation of the fluorophore from the quencher and allowing it to fluoresce.

Molecular beacons combine the specificity of a probe-based chemistry with the versatility of a reversible binding reaction.

2.4 System overview

PrimeQ is a real-time PCR unit into which the operator places a 96-well plate containing the samples to be analysed. The unit is connected to a PC via the USB port on which the application software (Quansoft) is run. The software allows the user to:

1. **Create** a PCR program defining dyes, read points, thermal cycling and analysis methods.
2. **Run** the program.
3. **Analyse** the fluorescence readings.



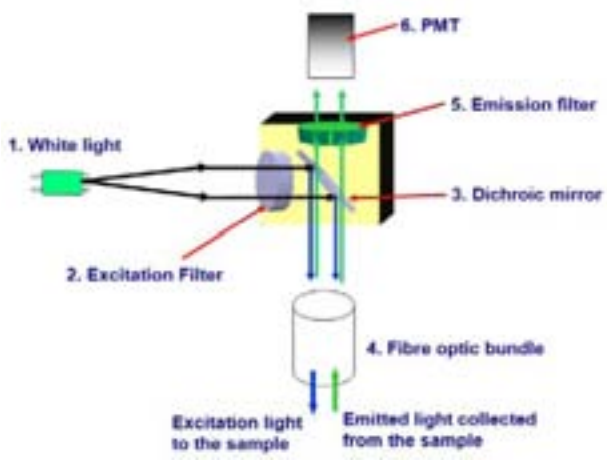
- **Thermal cycler:** The specialized block cycles the temperature according to the pre-defined PCR program.
- **Reader:** Allows fluorescence within each well of the plate to be read at specified points during the PCR program. The reader excites the samples at one wavelength and then detects the emitted fluorescence at another.
- **Software:** The unit requires a PC installed with the application software, Quansoft, for protocols to be defined and downloaded and for the data to then be analysed.
- **Consumable:** The unit accommodates a low-profile 96-well plate (skirted or non-skirted) sealed with optical sealing film.

PrimeQ consists of three key parts: the optics module; a thermal cycler module and a scanning module.

2.5 Optics module

The role of the optics module is to deliver excitation light into each well and measure the emitted light at defined points in the PCR cycle. The light source is a solid state white light source with the excitation and emission wavelengths controlled by a system of filters and a dichroic mirror housed in the user-changeable filer cartridges. Four such filter cartridges can be accommodated at any one time in the filter carousel.

2.5.1 The optical light path



Excitation light from the solid state white light source (1) passes through a light path and enters the filter cartridge. Only light at the desired wavelength is allowed to pass through the excitation filter (2).

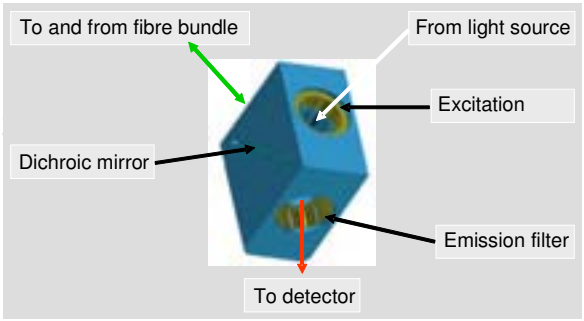
The light is then reflected through 90° by a dichroic mirror (3) and passed into a fibre optic bundle (4). From there, the light is directed into the wells of the sample plate by the fibre optic bundle, which excites the fluorophore present specific to that wavelength.

Light emitted from the sample well passes back up the fibre optic bundle, straight through the dichroic mirror (3) and then through the emission filter (5).

This filter blocks all wavelengths of light except the desired emission wavelength, which is passed onto the photon counting photomultiplier tube (PMT) detector (6). Fluorescence levels are then passed from the instrument to the PC via a USB connection where Quansoft analyses the data using algorithms. Results can then be exported, printed or displayed graphically.

2.5.2 Filter cartridges

Each set, comprising excitation filter, emission filter and dichroic mirror, is mounted in a cartridge. As detailed above, the role of the filter cartridge is to separate the excitation and emission spectra appropriate for the fluorophore being used. The excitation filter transmits only those wavelengths of light that excite the fluorophore, while the emission filter transmits only fluorescence produced by the sample. The emission light is always of a longer wavelength (more to the red region of the light spectrum) than the excitation light. As the filter carousel can hold multiple cartridges, the user is able to change the filter to suit the chemistry being used – a procedure that is easily performed from within the application software. Quansoft allows the user to install four filter cartridges in PrimeQ.



To allow the PrimeQ instrument to be optimized for the use of specific fluorophore combinations, the user can choose to adjust the light intensity of the LED light source (50, 100 or 200mA settings). This is useful for obtaining balanced results when using both bright and weak chemistries/dyes in the same experiment.

2.6 Thermal cycling module

The thermal cycling module is an 8 Peltier thermal cycling block with quad circuit technology into which the user places a low-profile 96-well sample plate. It is programmed from the application software and is able to reach any temperature between 4° and 98°C.

PrimeQ's thermal cycler block. accommodates a low-profile 96-well plate.

Each block is internally calibrated so that if the block is replaced, the temperature performance is unchanged without having to recalibrate the instrument

The thermal block fits into and can be accessed by opening the PrimeQ sample drawer. This provides the user with access to the block so that plates can be loaded or unloaded or the block removed for cleaning or replacement.

2.7 Scanning module

The scanning module delivers excitation light to the sample wells and then collects the emitted light. It also plays a role in heating the top of the sample plate and in preventing sample evaporation. It is made up of two basic components: the heated lid and the X-Y fibre optics.

- **Heated lid:** This component is made up of a heated glass plate that comes into contact with the top of the sample plate. Designed with a special optical coating, the heated glass plate provides uniform heating at the uppermost surface of the sample plate and thereby prevents sample loss due to evaporation and condensation. The heated lid also helps to hold the sample plate flat, and by being located within a cover, it provides a light-tight chassis for the X-Y fibre optics.
- **X-Y fibre optics:** Excitation light is carried from the optics module to the sample wells through a flexible fibre optic bundle. The X-Y mechanism scans each well in turn to transport the light into and out of the sample wells. The same fibre optic bundle carries the emitted light back from the well and into the optics module for delivery to the detector.

3 Quansoft

Using Quansoft

About this chapter

Accompanying PrimeQ is the intuitive, wizard-based software, Quansoft. This chapter discusses the concepts behind this software and looks at its application in the setting up and running of a real-time PCR experiment.

Read the first section for an overview of Quansoft in general terms and a brief discussion of how the various parts fit together. The next section helps the user to navigate around the software and reviews its basic functions and commands. The final section gives a simple step-by-step guide to the setting up, running and monitoring of a real-time PCR experiment.

3.1 Introducing Quansoft

PrimeQ is a real-time-PCR unit containing sophisticated thermal cycling and fluorescence detection technology. The user interacts with the system via Quansoft, the user-friendly Windows-based application software installed on a PC connected to the unit. The intuitive software allows the user to perform three main functions:

1. Create a PCR program and define fluorescent reading points.
2. Run the program.
3. Detect and analyse the fluorescence readings.

At the heart of Quansoft lie the Editors; powerful software features that have been specially designed to simplify setup, editing and customization of the experimental parameters. Plate layout, thermal cycling program and analysis method parameters can all be conveniently accessed from the Home page, with each Editor providing a flexible format and clear layout that makes viewing or changing any aspect of the protocol simple and straightforward.

Quansoft Home page

By employing a series of user-friendly windows, Quansoft enables any real-time PCR experiment to be created with ease.

Experiment Editor

This powerful Quansoft feature acts much like a shopping cart gathering together different elements of an experiment. Using combinations of plate layout and program parameters, it offers the user a flexible approach to the management of experiment protocols.

Program Editor

Individual cycling steps/stages or ramps are quickly added to build and display the thermal program. Fluorescent reads can be added at any step of a stage.

Plate Layout Editor

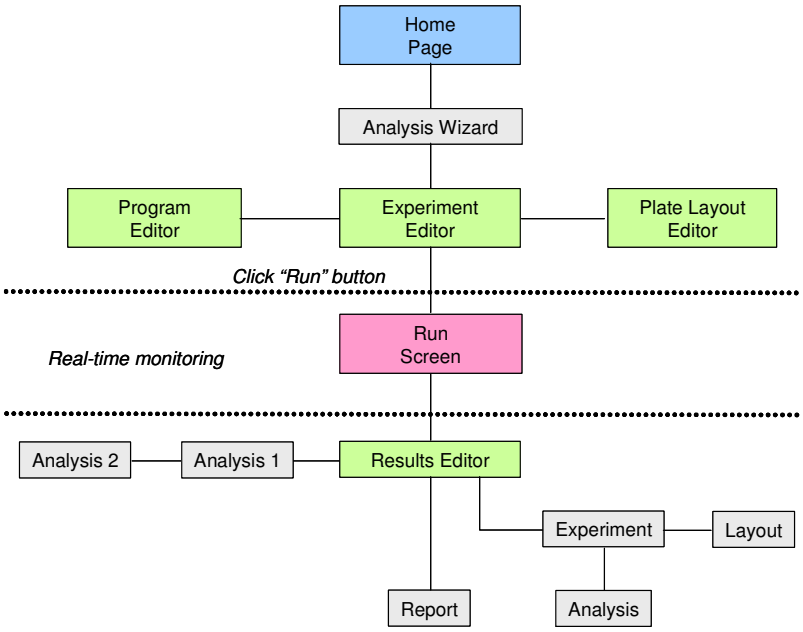
Within a matter of seconds, a 96-well microplate can be assigned with various sample types, including blanks, controls, standards or user-defined samples, all of which are colour-coded for ease of identification.

Results Editor

Results for each stage of the program containing fluorescent readings are shown on individual tabs and can be analysed separately using various analysis methods.

3.2 Software overview

The diagram below outlines the structure of Quansoft.



The starting point is the Home page, which provides easy access into the experiment setup. At the heart is the Experiment Editor, whose role it is to act as a 'shopping cart' for the plate layout and program elements. The Experiment Editor also incorporates the Analysis Wizard, which allows the user to define the method of analysis and possible report options to be applied to the results post-run.

3.2.1 Home page

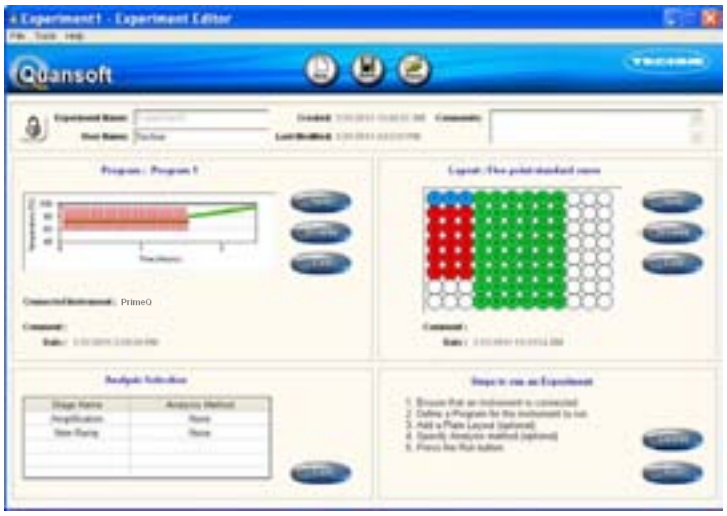
As shown in the schematic diagram above, the Quansoft Home page is the starting point for an experiment, providing quick links to all of Quansoft's most important features. Direct links into the different editor functions allow the user to quickly create a new experiment, re-run a previous experiment, edit a program or to analyse new or existing data. Basic maintenance and filter changing functions can also be performed from links on the Home page.

3.2.2 The Editors

Quansoft consists of four editors: the Program Editor; the Plate Layout Editor; the Experiment Editor and the Results Editor. These fit together to allow the creation, editing and analysis of a real-time-PCR run. The first three, as shown above, are part of the experiment setup and allow the user to set up the assay parameters. The Results Editor meanwhile, provides a platform by which to view the results and to change or specify parameters for the analysis post-run and report printout.

3.2.2.1 Experiment Editor

The Experiment Editor acts as a 'shopping basket' into which the components of the experiment are gathered i.e. the program, plate layout and the analysis parameters. It provides a summary of the setup thus acting as a gateway to browse, create or edit elements of the experiment. In essence, the role of this editor is to simplify the management of the experiment protocols and to provide a starting point for initiating a run on PrimeQ. Both the Plate Layout and Program Editors can be accessed from the Experiment Editor, and when combined with a set of analysis parameters, will produce a report of the results once the data has been collected. A copy of the experiment can be stored as a template thus allowing the easy routine analysis of a specific project. Experiments may also be shared between users with data from each user stored individually as a Results file. These files are saved in a **.qres** format.



Quansoft's Experiment Editor acts as a 'shopping basket' for the components of an experiment.

3.2.2.2 Plate Layout Editor

As its name suggests, the Plate Layout Editor defines the plate layout for an experiment. The 96-well plate can be left empty or assigned with blanks, controls, user-defined standards or test samples, which are all colour-coded for ease of identification. If need be, the well layout can be set up or changed after the run has completed and the data for analysis can be included/excluded using the right click flagging function.

Quansoft's Plate Layout Editor is a flexible platform for defining a plate layout.

In this editor, it is possible to set the name and value of samples used for a standard curve. When placing standards in the layout, the user will be asked to fill in the value of each standard in the Well Information table. To assign units to these standards, such as copies/well or ng DNA, options are available in the drop-down list, or a custom unit can be defined. As with the experiment files, a copy can be stored as a template saved in a .**qpla** format.

3.2.2.3 Program Editor

The Program Editor is the part of Quansoft where the user can define the temperatures, hold times and reading parameters to be used in the PCR run. The minimum requirement in order to start a run on PrimeQ is for the experiment to have a valid thermal cycling program – other settings such as plate layout and analysis method can be defined and/or changed post-run. These files can be saved to the Program Library in a **.qprg** format.

Quansoft's Program Editor provides a simple approach to defining the thermal cycling program and dye reads.

3.2.2.4 Results Editor

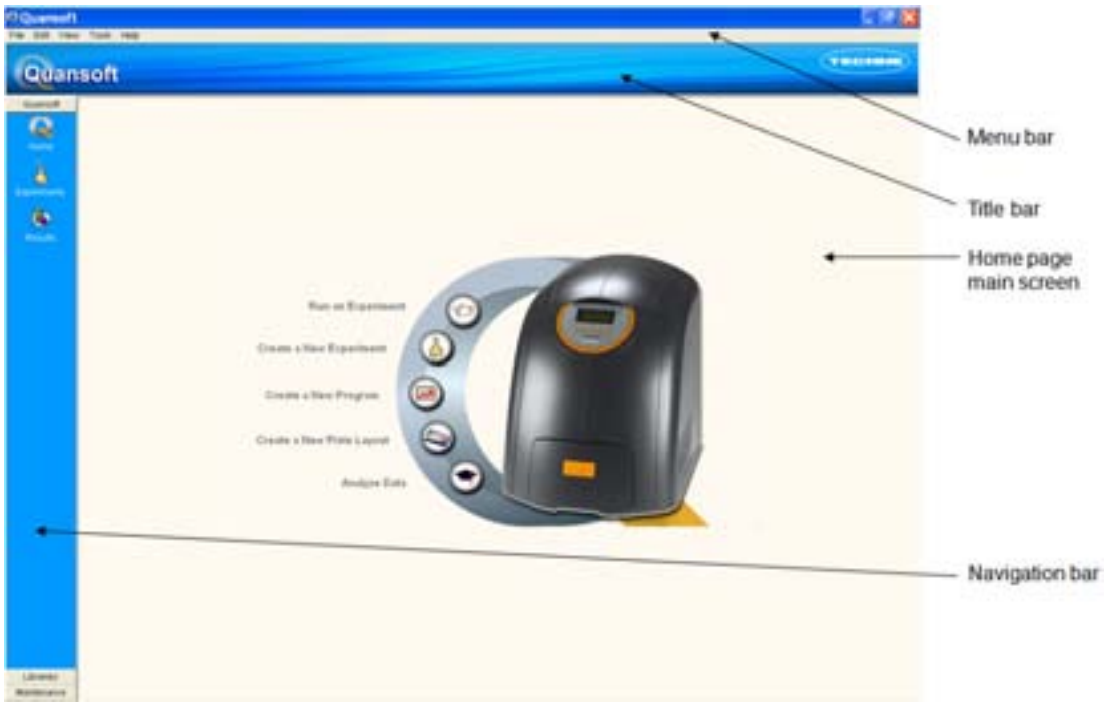
This editor displays the results data. Clicking on the Experiment tab allows analysis settings to be redefined or report options to be changed. The role of this editor will be examined more closely in Chapter 4.

Quansoft's Results Editor displays the results of the run, which can be sent to report or re-analysed with different parameters.

3.3 Using Quansoft

3.3.1 Home page

The Quansoft Home page acts as a gateway to the software functions, providing quick and easy links to both the Editors and their associated libraries. Other functions, such as basic instrument maintenance, filter changing, and administrative tasks, can also be carried out from here.



3.3.2 Main screen

Shortcuts for experiment setup and data analysis:

Run an experiment: goes to the Experiment folder to select an experiment.

Create a new experiment: goes to the Experiment Editor from where the user can create a new experiment or edit an existing one.

Create a new program: goes to the Program Editor from where the user can create a new program or edit an existing one.

Create a new plate layout: goes to the Plate Layout Editor from where the user can create a new layout or edit an existing one.

Analyse data: goes to the Results folder from where the user can open up the results of an experiment in the Results Editor.

3.3.3 Navigation bar

Located on the left-hand side on the Home page, the navigation bar provides easy access to the different editor libraries and the maintenance section of the software.

Home page icon:

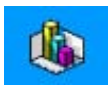
Clicking the **Home** icon when in one of the libraries returns Quansoft back to the Home page.

Library icons:

Clicking the **Program** icon opens the Programs directory in the library folder. The Program Editor will open if the **New** button is pressed.

Clicking on the **Plate Layout** icon opens the Layouts directory in the library folder. The Plate Layout Editor will open if the **New** button is pressed.

Clicking the **Experiment** icon opens the Experiments directory in the library folder. The Experiment Editor will open if the **New** button is pressed.



Clicking the **Results** icon opens the Results directory in the library folder. The Results Editor will open if a results file is opened.

Maintenance icons:

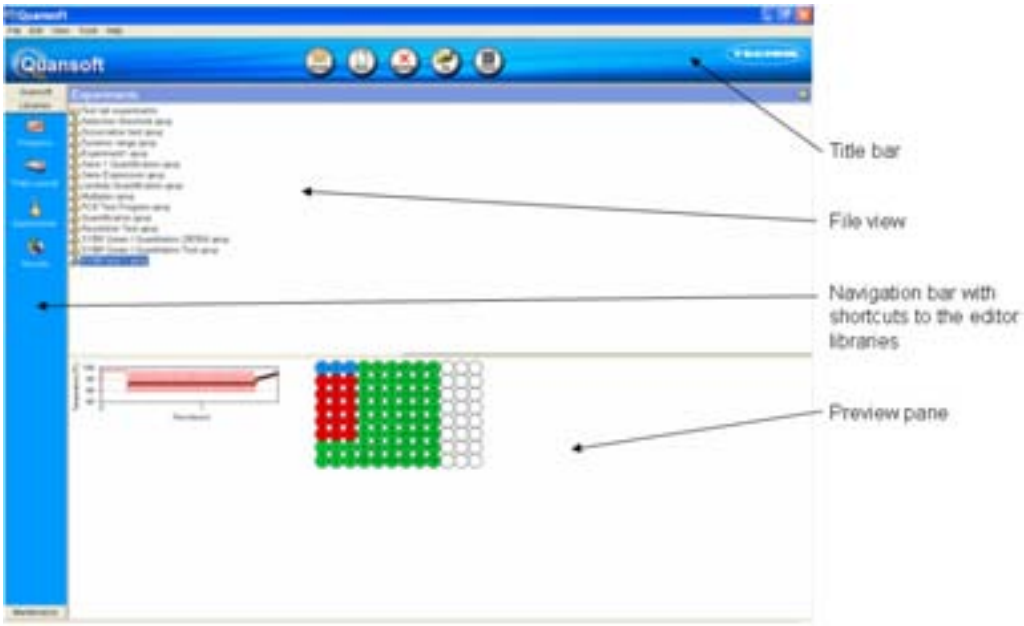
Clicking the **Filters** icon opens the Cartridge Access and Editing screen to allow the installation, changing and naming of filters using the Filter Wizard.

Clicking the **Security** icon allows customizing of the supervisor password (required for the administrative functions which are accessed under the **Instrument** icon).

Clicking the **Instrument** icon will allow the user to view instrument-specific details including, for example, the instrument serial number and block cycle count. The default supervisor password is techne - we suggest that you change this as soon as possible. Please ensure that you keep a record of the password as without it you will not be able to access these functions.

3.3.3.1 Library view

Clicking on the **Libraries** tab, the navigation bar will display icons for each library: Programs (.qprg files), Plate Layouts (.qpla files), Experiments (.qexp files) and Results (.qres files). The contents can be browsed and previewed in the pane in the lower half of the screen, and by opening it up in the relevant editor (either by double-clicking the file or browsing from within the relevant editor), the files can be edited and analysed with ease. The libraries can be navigated by moving up and down the directory structure with users able to create folders and organize data in the usual way.



3.3.4 Title bar functions

Shortcuts to frequently used library functions are also found on Quansoft's Title bar.

The **Home** button returns the software to the Home page where there are quick links to the other functions.

The **New** button will open a blank editor window depending on which library has been selected (inactive for the Results library).

The **Delete** button can be used to delete selected files or folders from a library.

The **Up** button allows the user to navigate back to a previous folder (used if the directory structure has been turned off).

The **View** button will change the way the files are displayed in the file view pane (detail, large/small icon, list views).

The following buttons are displayed on the Title bar when using one of Quansoft's Editors:

The **Finish** button (Results Editor) saves the changes made and closes the editor.

The **Save** button will open the Save As window, allowing the user to save the file under a new name.

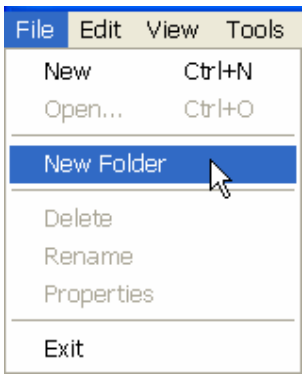
The **Open** button allows the user to browse and select files from a library.

3.3.5 Menu bar functions

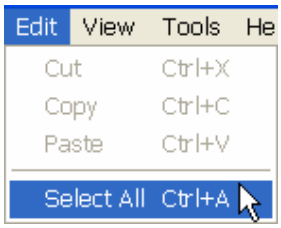
Quansoft's menu bar offers many of the functions found in a typical MS Windows application.

- Click the Quansoft icon to move, maximize, minimize or restore the screen.

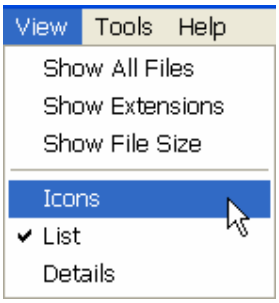
- Click on the menu bar options beneath the Quansoft icon for drop-down menus offering the following functions:



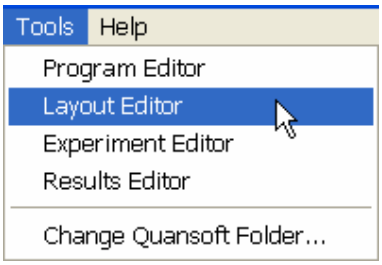
File: Contains file manager commands. Click the appropriate function to open, close, delete, re-name or to check the properties of a particular file. Greyed-out commands are disabled.



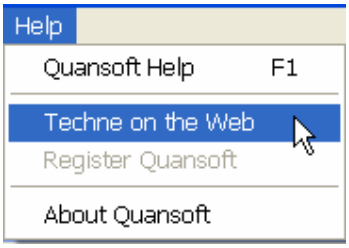
Edit: Contains functions for the cutting, copying or pasting of selected text or graphics.



View: Allows the user to change the way the files appear in the file view. Choose to display simply as icons or display with further details such as file name and size.



Tools: Provides direct access into the editor of choice - simply click on the editor name. The **Change Quansoft folder…** command allows the user to change the destination of any saved files. Quansoft will automatically save all files to My Documents\Quansoft; change the destination here if required.



Help: Off- and on-line resources. Click **Quansoft Help** (short cut key F1) for access to this Operator guide, or click on **Techne on the Web** to link to the Techne website.

Clicking on **About Quansoft** will bring up details about the software including the version and serial number. Registered users of Quansoft will be kept informed of software updates.

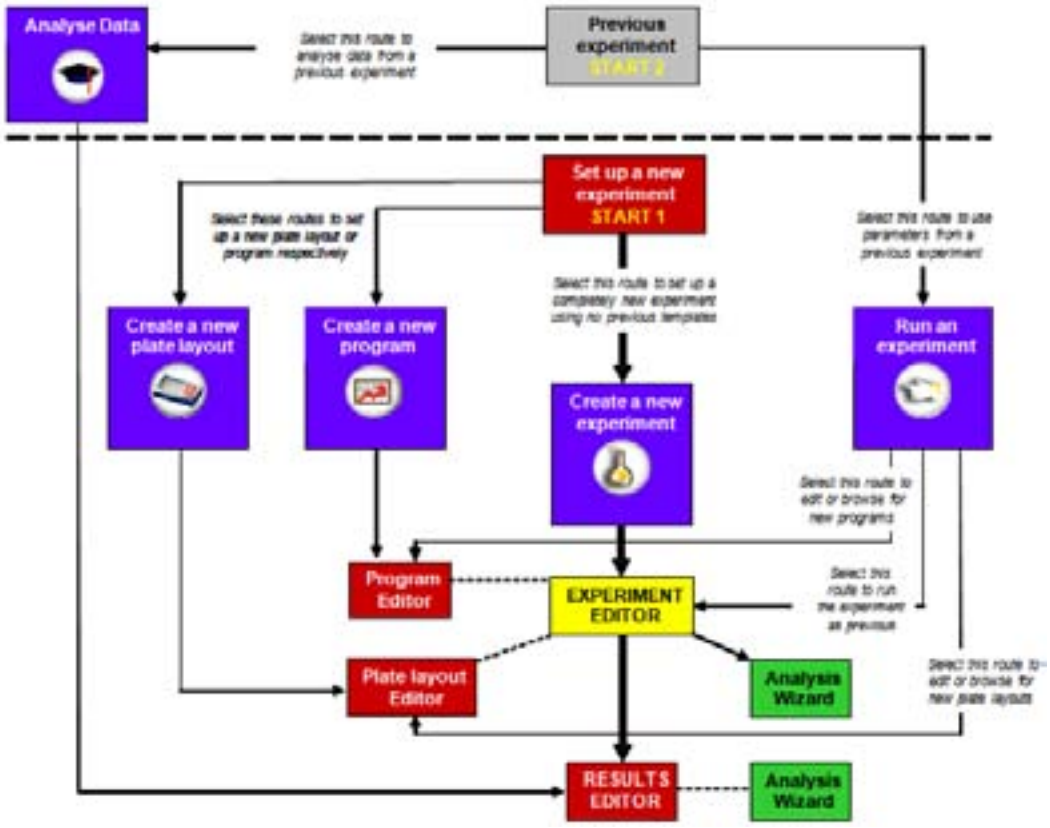
3.3.6 Accessing the editors

The user can access the Quansoft editors by the following routes:

- **Home page:** Click the round shortcut icons as discussed in section 3.3.2.
- **Menu bar:** Go to **Tools** and click on the editor of choice.
- **Library files:** Clicking on a library file will open up that file within the relevant editor. Either browse a library file from the Home page or browse from within an editor.
- **New or Browse**: Clicking on these buttons in the program or plate layout pane of the Experiment or Results Editor opens up the relevant editor within. Files can then be browsed, edited or created from new.
- **Start menu:** Click on the Windows **Start** button in the bottom left hand corner. Choose **All Programs/Techne/Quansoft/Editors** and then select the editor of choice.

3.4 Setting up an experiment

3.4.1 An Overview



Schematic diagram showing the options available for setting up an experiment on PrimeQ.

The icons in the blue boxes represent the shortcut buttons available from the Home page.

START 1: Choose to set up an entirely new experiment in the Experiment Editor, browse or edit previously saved plate layout or program templates or else access the Plate Layout and Program Editors direct from the Home page.

START 2: A previous experiment can be opened and run with no changes to the parameters or results from a previous run can be analysed directly.

3.4.2 Creating a new experiment

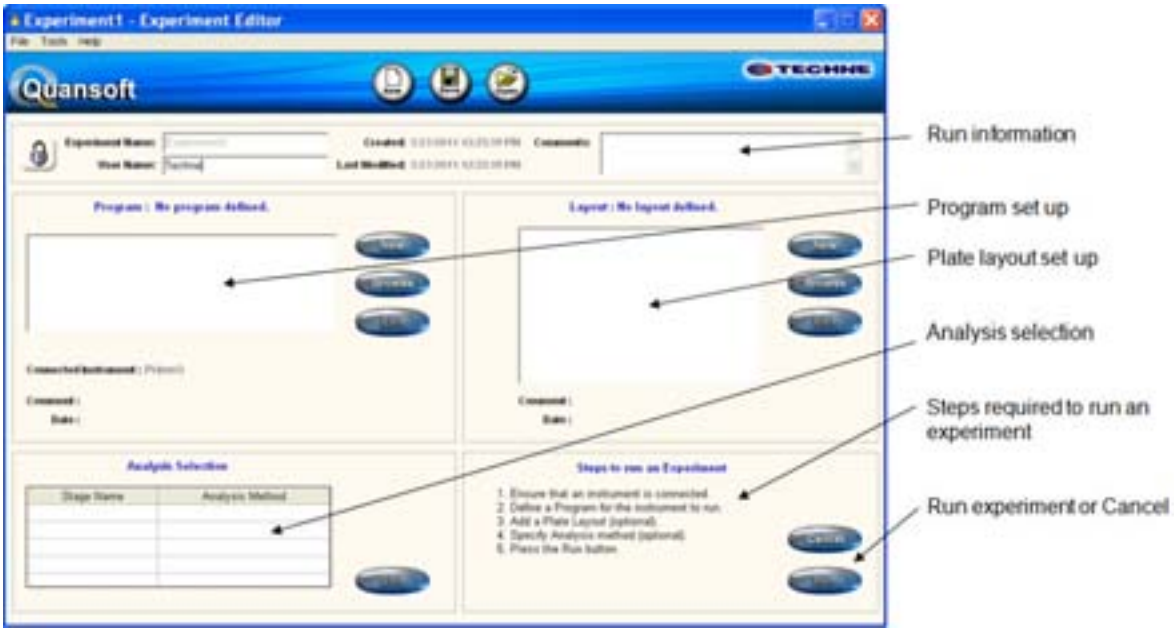
- From the Home page click on **Create a New Experiment**.

A blank experiment template will open in the Experiment Editor.

The Experiment Editor allows the user to perform three important functions:

- Define a program,
- Define a plate layout
- Define an analysis method

Note: only the setting of a program is essential for a run to be performed; plate layout and analysis method can both be defined after the run is complete.



The options for setting up individual experiment components are:

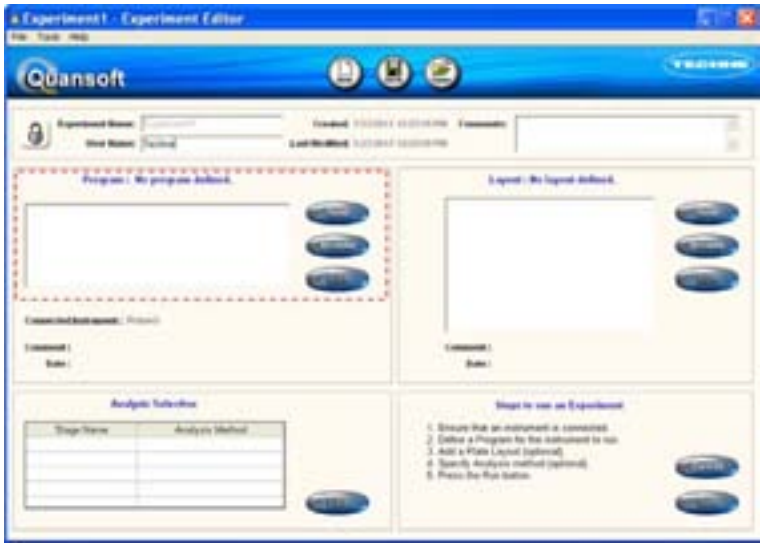
1. Program Setup:
    a. From **New**
    b. **Browse** an existing template
    c. **Edit** the selected template

2. Plate Layout Setup:
    a. From **New**
    b. **Browse** an existing template.
    c. **Edit** the selected template
3. Analysis Method Selection

This chapter will look at each of these functions in turn.

3.4.3 Setting up a program

The PCR program is defined using the top-left area of the Experiment Editor main screen:



There are three ways to define a PCR program:

Click this button when the program is to be defined entirely from new.

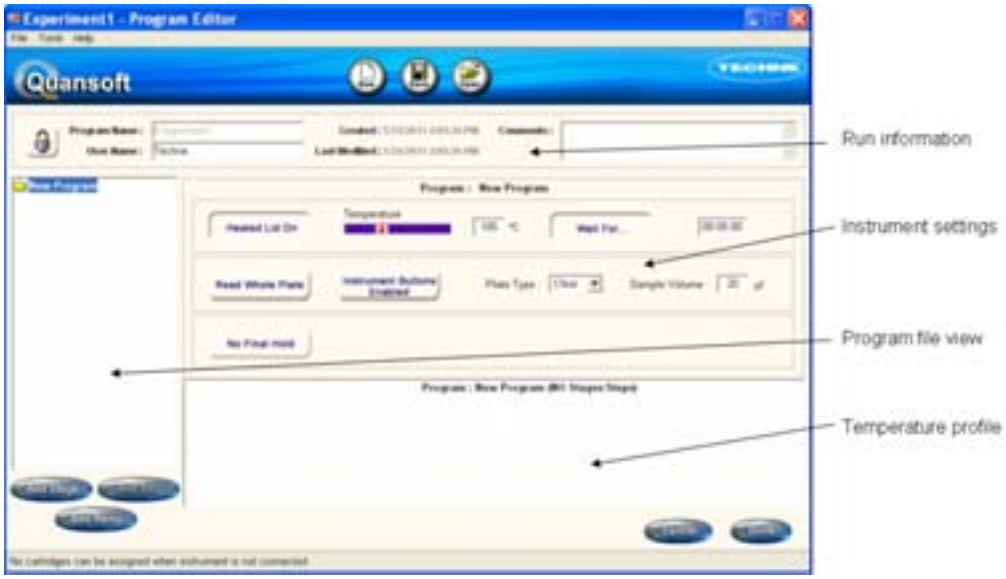
Click **Browse** to search the Program library files for existing templates.

Click to edit the selected program template

3.4.3.1 Define a new program

- To create a new program, click the **New** button.

The Program Editor will open up within the Experiment Editor with a blank program screen.



- **Run information:** The user can enter a user name and add any other comments relevant to the program.
- **Instrument settings:** User-defined settings for heated lid temperature, disabling of the instrument buttons, plate type, sample volume and whether to read entire plate or include a final hold in the PCR program.
- **Program file view:** Displays the structure of the program thereby providing the user with a clear view of the overall protocol.
- **Temperature profile:** Provides a summary plot showing the temperature profile for each cycle of the stage (or the complete program when viewing at the Program level).

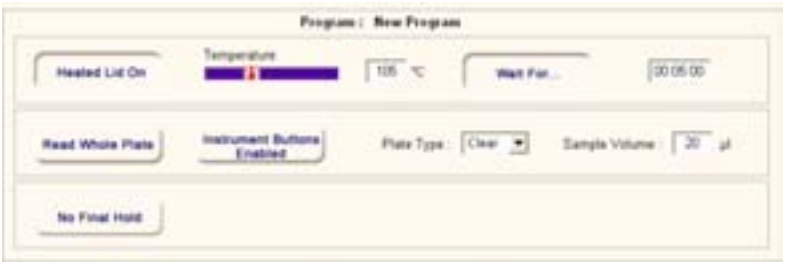
Clicking on the padlock icon to a **locked** position prevents the user from accidentally saving over the existing file – to save the changes the user must use the **File** menu **Save As**… option and save the file under a different name.

3.4.3.2 Run information

- Enter a user name and any comments in the appropriate text boxes (optional).

3.4.3.3 Instrument settings

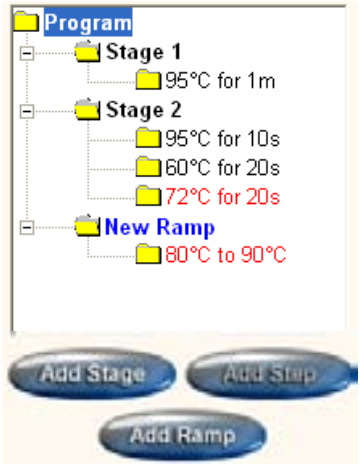
- Define the general instrument settings for the run.



- **Heated Lid:** Default 105℃. Set a temperature (possible temperature range from 100℃ to 115℃).
- **Wait For…**: Set a 'wait for' time (i.e. the time to wait while the lid heats up before the block thermal program begins, hrs:min:sec). Or click the button to wait until heated, the program will then start as soon as the lid reaches the set temperature.
- **Read Whole Plate:** Default **ON**. Click to **Read only filled wells**. This can be useful to reduce the time taken to read the plate if only a partially filled plate is in use. Note: a plate layout **must** be defined in order to read only the filled wells.
- **Instrument Buttons:** Default enabled. Click to disable (prevents against accidental stoppage of the instrument using the instrument buttons, so that the program can only be controlled from within the software).
- **Plate Type:** Default **Clear**. Scroll down to select a different plate colour.
- **Sample Volume:** Default **20µl**. Change as appropriate (max 50µl, min 15µl).
- **Final Hold:** Default **OFF**. Activate by clicking. Set time and temperature as appropriate.

3.4.3.4 Program

A program is set up in a logical fashion whereby temperatures and hold times are defined for each stage, as would be done on a typical thermal cycler. The major difference is that the user will tell the instrument when to take a fluorescent reading and which filter cartridge(s) should be used.



Each stage of the Program is made up of temperature steps cycled x number of times.

- Click **Add Stage** to open a new stage and label it accordingly.
- Click **Add Step** to add the relevant temperature steps to each stage.
- Click **Add Ramp** to add a ramp read (melt stage, see section 3.4.3.6).

Steps with a dye read are highlighted in red; a ramp read is highlighted in blue.

Level 1: Program

- Name the program if desired (the default is **New Program**) and set the instrument settings.

Level 2: Stage

- Click **Add Stage**. On doing so, a new stage folder will appear in the file view:

The default name **New Stage** can be changed if required.

When a stage name is highlighted in the program file view, the stage parameters box in the centre of the screen allows the general parameters for that stage to be defined.

- **Heated Lid:** The default is to keep the heated lid temperature unchanged. To change, click the button and a time and temperature setting box will appear adjacent (possible temperature range from 100℃ to 115℃).
- **Number of Cycles:** Define how many times the stage should cycle (range between 1 and 99).
- **Do Not Pause:** Default **ON**. If a pause is required after this stage then click on this button.

To delete or rename a stage, right click on the stage folder and select the appropriate command. Alternatively, highlight the stage and select the appropriate command from the **Tools** menu in the tool bar.

Level 3: Step

- Click on **Add Step** to add a temperature step to a stage.

When a step is highlighted in the program file view, the step parameters box in the centre of the screen allows the parameters for that step to be defined.

- **Temperature:** Set using the sliding thermometer or type a value in the box.
- **Hold Time:** Shown in hrs:min:sec up to a maximum of 99:59:59 (minimum 00:00:01). The default setting is **seconds**, therefore for a time in seconds, type in the number of seconds and press the enter key. To set the time in hours, add “h” after the numerical value, e.g. 1h, and for minutes add “m”.
- **Number of Reads:** Displays the number of readings programmed for the step (maximum of 4).
- **Add Read:** Fluorescence reads can be added by clicking on the **Add Read** button. See section 3.4.3.5 for details of adding a read to a step.
- **Inc/Dec Temp:** Used in programs where an incremental increase or decrease in temperature is required. This function is useful in ‘Touchdown’ PCR, for example, where the annealing temperature is kept high in the initial cycles and then gradually brought down with the aim of reducing non-specific amplification.
- **Inc/Dec Time:** Used in programs where an incremental increase or decrease in time with each cycle is required such as long template PCR.
- **Max Heating Rate**: Allows the heating rate to be set between 0.1°C/sec and 2.2°C/sec.

Note: If any special parameters are selected, including Inc/Dec Temp or Inc/Dec Time, then a blue triangle will appear in the temperature profile plot.

To delete a step, right click on the step and select **Delete**. Alternatively, highlight the step and select **Delete** from the **Tools** menu in the tool bar.

3.4.3.5 Adding a read to a step

The user must tell the instrument at which point in the thermal cycling program to collect fluorescent readings and which filter cartridges to use. Reads can either be added at the same time as the step parameters or added stage-by-stage after the thermal cycle program has been completed.

- To add a read to a step, highlight the step to display the step parameters box and click on **Add Read**.

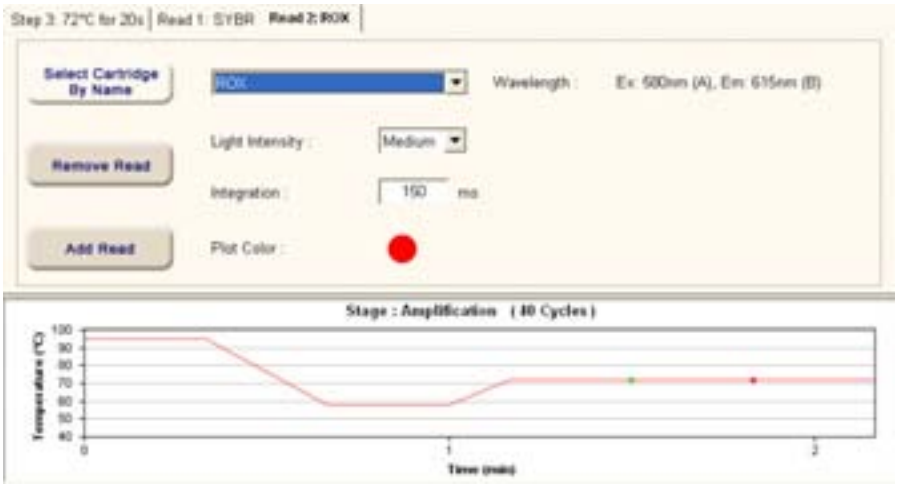
The filter programming options will then be displayed:

- **Select Cartridge by Name/Wavelength**: This is the name given to the cartridge on filter installation and is stored in the instrument memory. The filter cartridge can either be chosen on the basis of its name (as given during filter installation) or by its ID wavelength. Click to

change between these options. If the user is creating a new protocol and the instrument is not connected, it is not possible to select filter cartridges (the drop-down box is greyed-out). The user will be prompted to choose the filters on connection to the instrument or when trying to run the program.

- **Light Intensity**: Choose low, medium or high depending on the type of fluorophore in use and its concentration. Default: **Medium**.
- **Integration**: This is the length of time during which fluorescent data is collected for each well. This may need to be increased for weaker dyes or where there is a high level of background fluorescence present in the chemistry. Increasing the integration time in the latter case can improve the signal-to-noise ratio. The integration time can be changed from 250ms for weak dyes down to 50ms for stronger fluorophores (the default is set at 150ms).
- **Plot Colour**: The colour displayed will depend on the selected filter cartridge and will default to the colour which was chosen for that particular filter during installation. Double-click to bring up other options.
- **Remove Read**: As the name suggests.
- **Add Read**: If another dye read is to be added (up to four dye reads can be added per step), click here and repeat the process choosing the filters appropriate to the second dye. The reads will be tabbed at the top of the settings box allowing the user to easily navigate between them.

Repeat this procedure, adding reads where appropriate. The temperature profile graph will depict the read points in the assigned colour.



Separate reads appear as individual tabs in the settings box; simply click to view or change the parameters.

Any stages assigned a read will be shown in red in the program file view and the read will be represented on the thermal profile plot by a circle in the chosen plot colour.

3.4.3.6 Setting up a ramp read stage

The **Add Ramp** button allows setup of a dissociation (melt) curve stage whereby the temperature is raised in small increments between a defined range of temperatures; typically from the primer annealing temperature up to the system denaturation temperature of 95°C.

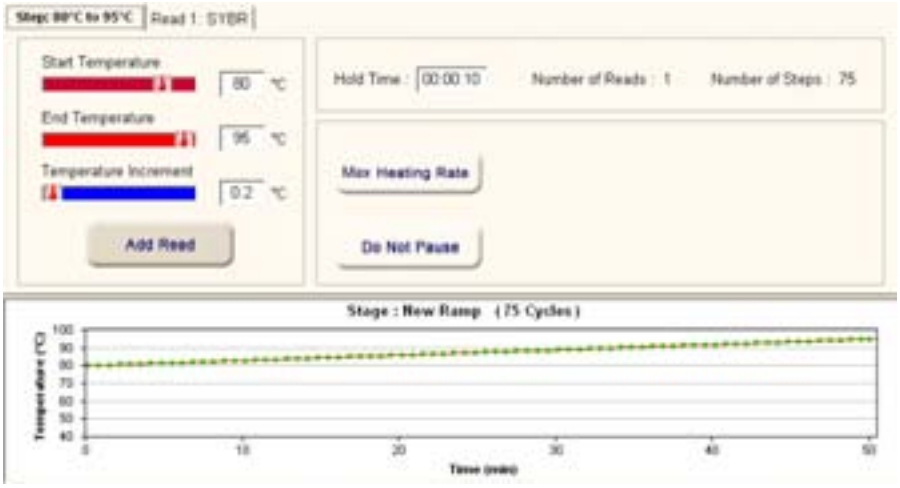
By taking fluorescence readings at each temperature increment and then plotting a dissociation curve, the point at which the dsDNA template melts into two strands can be identified. This procedure is useful in product identification (the exact dissociation temperature is a characteristic of the GC content, length etc.) and is commonly performed at the end of a PCR run where an

intercalating dye such as SYBR® Green I is used, for confirmation that the correct product has been amplified.

- Click **Add Ramp** to open the ramp read parameters box:

- **Start Temperature:** Starting temperature of the ramp can be anywhere from 4°C.
- **End Temperature:** The temperature can be set to ramp to any temperature up to 98°C.
- **Temperature Increment:** The temperature can be increased in increments of between 0.1 and 5°C. The default is 0.5°C.
- **Hold Time:** The user can choose run the ramp with a set hold at each temperature; this can be any time up to a maximum of 99:59:59 hrs:min:sec. The default is 10 seconds.
- **Max Heating Rate:** Allows the heating rate to be set between 0.1°C/sec and 2.2°C/sec.
- **Do Not Pause/Pause After Stage:** If set to pause, the run will pause after each stage; click to change to Do Not Pause.
- **Number of Reads:** Displays the number of readings programmed for the ramp (maximum of 4).
- **Number of Steps:** Displays the total number of steps for the ramp stage. This will depend on the temperature range and increment.

- Set the start temperature, the end temperature and the temperature increment between reads. These can be changed either by using the sliding thermometer or by typing in the respective box and clicking the enter key. The number of steps will be shown in the top-right according to the settings chosen.
- Set the hold time and choose whether to set the heating rate or to program in a pause.
- Add a read to the ramp stage by clicking **Add Read** in the ramp read parameters box. The same principles apply for setting up a read in a ramp stage as for a typical temperature step, described in section 3.4.3.5.



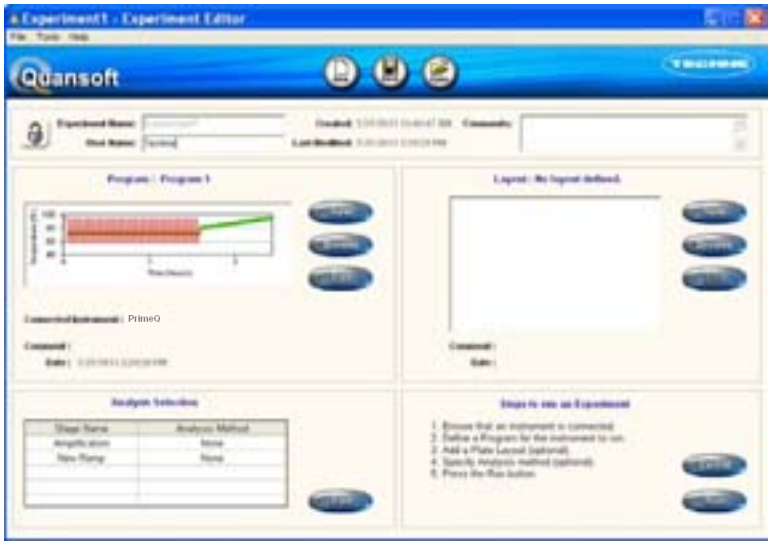
The read appears as a separate tab labelled according to the dye name. The temperature profile plot shows the reads as colour-coded points.

Once the program is complete, clicking on the program name at the top level in the program file view will show the complete thermal plot:

Any parts of the program, such as temperatures, hold times, read points, filters etc. can be edited at any point prior to the run.

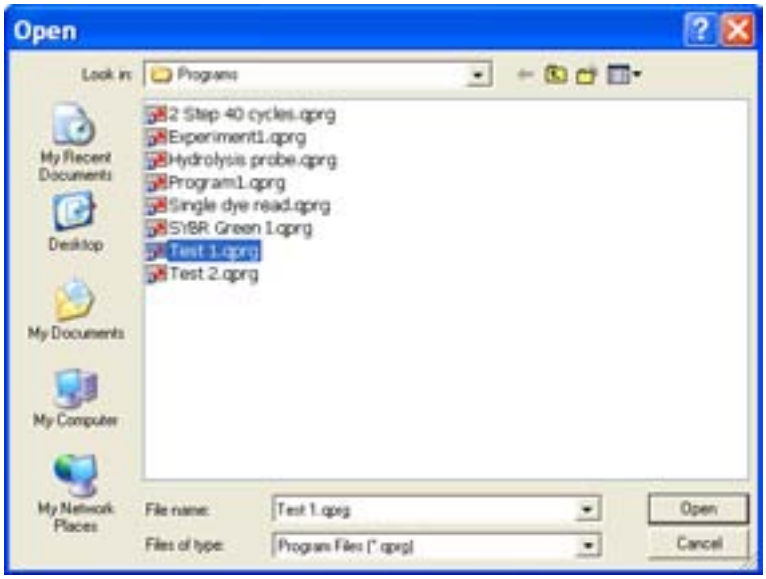
- Click on **Save** to save the program to the Program Library.
- Click **Done** to complete the program and return to the Experiment Editor.

The program will appear in the program pane of the Experiment Editor:



3.4.3.7 Browse for an existing program

The user can browse for an existing program file by clicking on the **Browse** button in the Program pane of the Experiment Editor. This will open up the Program library folder and display any existing templates (.qprg files).



- Highlight a folder and click **Open** to open up the program file in Experiment Editor. The template is ready to use.

3.4.3.8 Edit a program

A program already present in the Experiment Editor can be edited simply and easily by clicking the **Edit** button in the Program pane of the Experiment Editor. Clicking the **Edit** button will open up the program within the Program Editor thus providing all the same functionalities as if defining a program from new. Any of the thermal cycling or read parameters can be defined or changed from here.

- Click **Done** to return to the Experiment Editor and the edited program will appear in the Program pane.

3.4.3.9 Create a new program from the Home page

Clicking on **Create a new program** from the Home page provides a quick link through to the Program Editor. Define the thermal cycling parameters, the filter cartridge settings and the read points as detailed in section 3.4.3. The program must be saved to the Program Library to become available for browsing.

3.4.3.10 Saving a program to the library folder

The program file can be saved at any point during the setup using typical Windows commands.

However, if the padlock icon in the top-left of the Editor screen is locked, the current file in use is designated **read only**. This provides a useful tool to protect against the accidental over-writing of files meaning that this file would have to be saved under a different file name.

This function can be turned off by simply clicking the padlock icon or by choosing the **File** option in the menu bar and clicking on **Lock**.

IMPORTANT: Certain characters must not be used in the file name otherwise it may become corrupted and you may not be able to open the Results file. These characters are: < > & ‘ “

- On the menu bar in Program Editor, select **File** and then **Save As…**

- Change the file name if required. The file (.qprg format) will automatically be saved to the directory My Documents\Quansoft\Programs. Change the destination by browsing the file directories shown in the **Save in:** drop-down menu.

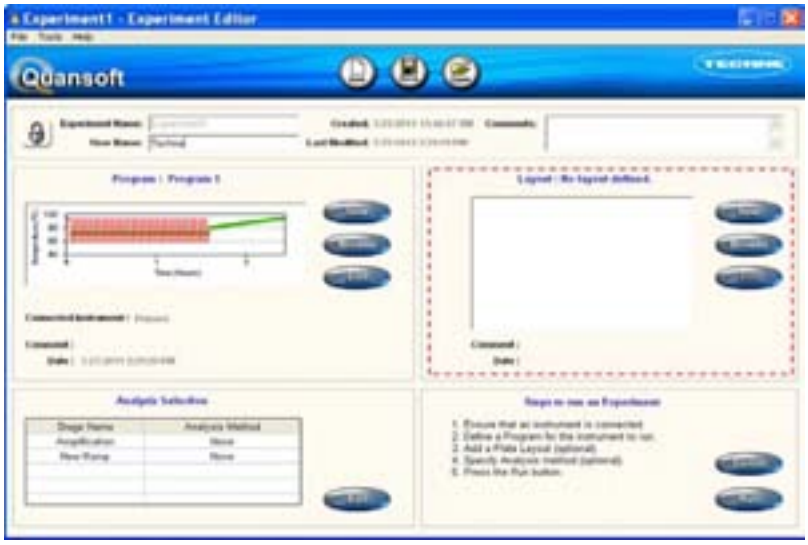
The library files can be accessed by browsing within the Program Editor as shown in section 3.4.3.7 or accessing the library folders from the quick links on the navigation bar of the Home page (detailed in section 3.3.3).

The destination of all Quansoft files can be changed using the **Tools** function located on the menu bar on the Quansoft Home Page.

- Click on **Change Quansoft Folder…** A box displaying the current destination folder appears.
- To accept this destination, press **OK,** or select **Browse** to change the location.
- Click **Cancel** to abort the action and return to the Home page.

3.4.4 Setting up a plate layout

The role of the Plate Layout Editor is to define what types of samples go in to which wells of the plate. It is represented in the top-right panel of the Experiment Editor. The plate layout can be cleared or changed before or after the run.



As with the program setup, the plate layout can be defined in three ways:

Click this button when the plate layout is to be defined entirely from new.

Click **Browse** to search the plate layout library files for existing templates.

Click to edit the selected plate layout template.

3.4.4.1 Creating a new plate layout

- To create a new plate layout, click the **New** button.

The Plate Layout Editor will open up within the Experiment Editor with a blank plate layout template.

- **Run information:** Add a user name and any comments as appropriate.
- **Sample types:** Shows the active sample types in use: No Template Control (NTC) Standard (STD) and Unknowns (UNK) are defaults. Clicking on the 'Sample type' icon takes the user through to a settings box, which allows other sample types to be chosen or for custom types to be created.
- **Next group/replicates:** Permits up to 9 replicates to be set and these replicates are then 'grouped'. To change the group name/order, adjust in **Next group** by scrolling down.
- **Well information:** As a sample is allocated to each well, information about the sample will appear in the adjacent table. A default name and number is assigned e.g. Standard 1, but this can easily be changed by highlighting and over-typing. Concentrations can be added for standards (units defined by the drop-down menu) and any comments added.

- **Show all wells:** The well information table will show only the details for the currently selected sample type, e.g. standards, unless this button is clicked. Click again to change back.
- **Reset names:** Allows any user-defined changes to the names to be returned to the default.
- **Function buttons:** Icons on the left-hand side provide easy access to basic functions (further details in section 3.4.4.6). Click once to activate.

3.4.4.2 Sample types overview

Before setting up a real-time PCR experiment, you must consider what types of samples are to be used in the assay. Sample types generally fall into the following categories:

- Standard (**STD**): Samples of known concentration are used to construct a standard curve in order to extrapolate unknown sample concentrations.
    - Standards should always be prepared carefully since the accuracy of a quantification assay can be no better than the accuracy of the standards.
    - Reduce the variability by using several points in the standard curve (at least three) and when producing a dilution series, use serial dilutions no more than one order of magnitude apart (1:10, 1:100 etc).
- Unknown (**UNK**): Samples containing an unknown quantity of the template being reported.
- No template control (**NTC**): Similar principle to a negative control but the missing component is the template. A NTC controls for contamination and false-positives. For assays not using an internal positive control (IPC), the calls for unknown sample can be based on a threshold determined by the NTC. The threshold determines the minimum fluorescent signal that must be achieved to assign a positive call to the sample.
- Negative control (**NEG**): Sample wells with one or more of the components of the reaction mixture missing. Useful in end-point assays as above and in checking for contamination and false-positives.
- Internal positive control (**IPC**): Particularly useful in plus/minus scoring. An IPC is a separate PCR reaction requiring a different set of primers, probe and template. The template is “spiked” into the PCR at a known concentration.
- Positive control (**POS**): Used to confirm that the PCR reaction is successful. Preferably uses the same template and primer pair as the unknown samples. Can be useful in end-point assays for defining the range of results.

- Calibrator (**CAL**): In relative quantification methods, the ratio of the DNA template in different samples may be normalized to a calibrator sample. Particularly useful when comparing Cq values (section 4.7).
- Reference Gene (**REF**): A common gene that is expressed in all samples at the same level. Can be useful in relative quantification methods.

3.4.4.3 Choosing sample types

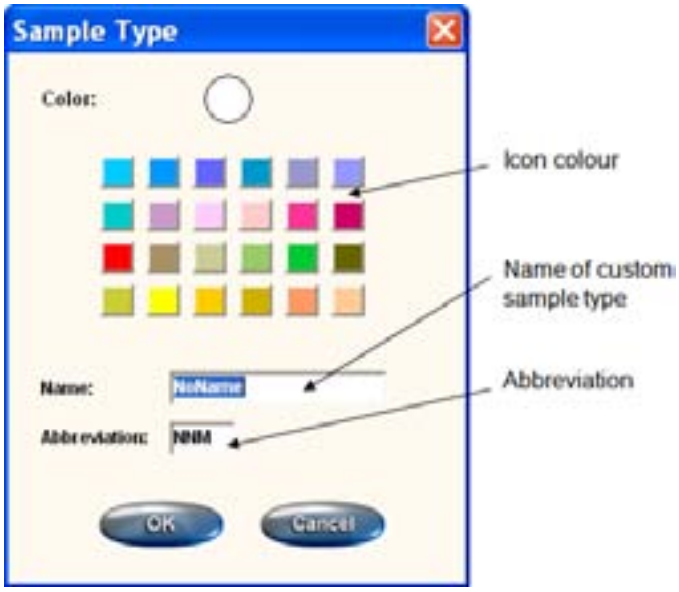
No Template Control, Standard and Unknown sample types are set as defaults in Quansoft, represented by the icons displayed above the plate layout.

Sample types: Use this function to choose and define different sample types. Click once to bring up the **Sample Types** box (this can also be accessed from the menu bar by clicking **Tools**/**Define Sample Types…**).

- To add a sample type to the **Active** list, simply highlight the sample type required from the **Available** list and click the left arrow to move from the available into the active window. This will then appear as an icon on the Plate Layout Editor main page next to the default icons for standard (STD) and unknown (UNK).
- To remove a sample type, simply highlight the sample name in the active list and click on the right-pointing arrow to transfer it from the active to the available list.

3.4.4.4 Adding a custom sample type

- From the Sample Types box, click on **Add Custom** and a settings box will appear.
- For the custom sample type, choose the icon colour and assign a name and abbreviation. Click **OK** when done.



The custom sample type will now appear in the available list. Move over to the active list using the arrow if the sample type is to be used in the current experiment. Close the sample types setting box and return to the Plate Layout main screen.

3.4.4.5 Replicates

Choose how many replicates there are for each sample from the scroll-down menu. The **Next group** added to the plate layout will run in numerical order unless otherwise specified.

3.4.4.6 Assigning sample types to wells

- Click on a sample type icon, for instance, the UNK icon, and the icon will become highlighted to show it is active.
- Click on or drag over the wells of the plate with the mouse and the corresponding colour and number will appear in each well.

Information about each well will appear in the table to the right as the wells are filled.

- Repeat with the other sample types, using the **Erase** or **Clear** functions (see below) to make amendments at any time, or press **Cancel** to abort the procedure and return to the Experiment Editor.

The function buttons provide various tools that can assist in assigning the plate layout by simply clicking on the icon with the mouse so that it becomes highlighted. Clicking again de-activates the function.

Erase: Clears individual wells that have been assigned a sample type.

Fill by row: Allows quick-filling of the plate row by row.

Fill by column: Allows quick-filling of the plate column-by-column.

Fill plate: Quick fill the remaining wells with the highlighted sample type.

Clear plate: Clears the plate of any assigned sample types.

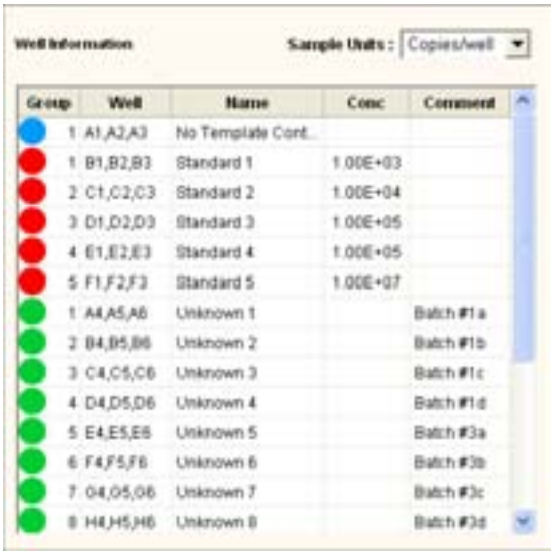
Undo: Cancels the previous operation.

Rotate plate 180°: Useful if the plate was loaded into the instrument the opposite way round to the designated layout.

These functions can also be accessed from the **Tools** option in the menu bar.

3.4.4.7 Well information table

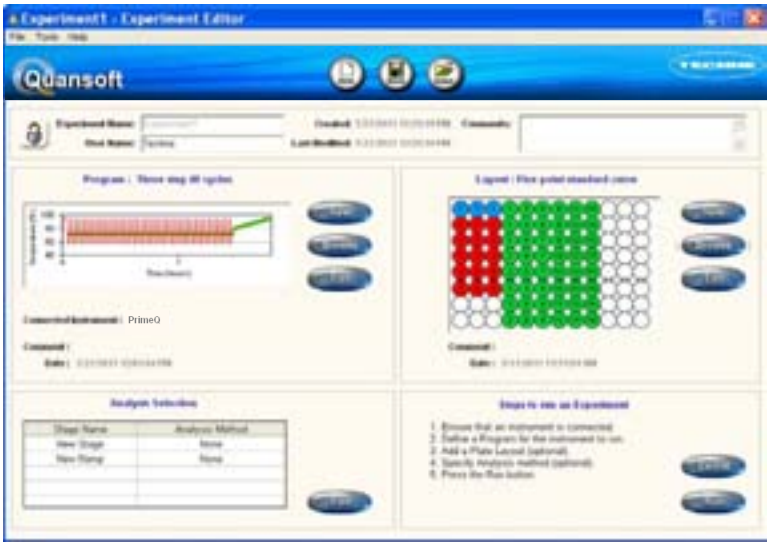
As a sample is allocated to each well, information about the sample will appear in the adjacent table. A default name and number is assigned e.g. Standard 1, but this can easily be changed by highlighting and typing over the text. Use the **Reset Names** function to return any user-defined changes to the default. Concentrations can be added for standards (units defined by the drop-down menu) and any comments added.



Choose to add user-defined names, concentrations and units of standards or any comments specific to a sample.

The Well Information table will contain a complete list of all the sample types in the plate, but to list the details of just a selected sample type, click the **Selected Sample Type** button and then click on a sample type icon to choose which sample information to display. For instance, to display only the well information for the standards, click on the Selected Sample Type button and then click on the red STD sample icon above the plate layout. When only the selected well information is being shown, the function button will now display **Select All Wells**. Click on this button to return to displaying the complete well information.

- Press **Done** when complete. Quansoft will return to the Experiment Editor and the new plate layout will appear in the plate layout pane. Press **Cancel** at any time to abort the procedure and return to the Experiment Editor.

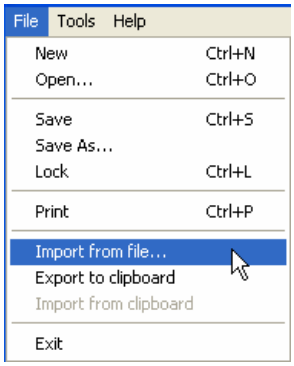


3.4.4.8 Importing/exporting plate layouts from/to Microsoft® Excel

Sample information can be copied into the Plate Layout Editor from an Excel spread sheet. The Excel file must be in the following format to permit correct import of sample types and well information:

	A	B	C	D	E
1	Well	Type	Name	Concentration	Comment
2	A1	NTC	No Template Control 1		
3	A2	NTC	No Template Control 1		
4	A3	STD	Standard 1	10	
5	A4	STD	Standard 1	10	
6	A5	STD	Standard 2	100	
7	A6	STD	Standard 2	100	
8	A7	STD	Standard 3	1000	
9	A8	STD	Standard 3	1000	
10	A9	STD	Standard 4	10000	
11	A10	STD	Standard 4	10000	
12	A11	STD	Standard 5	100000	
13	A12	STD	Standard 5	100000	

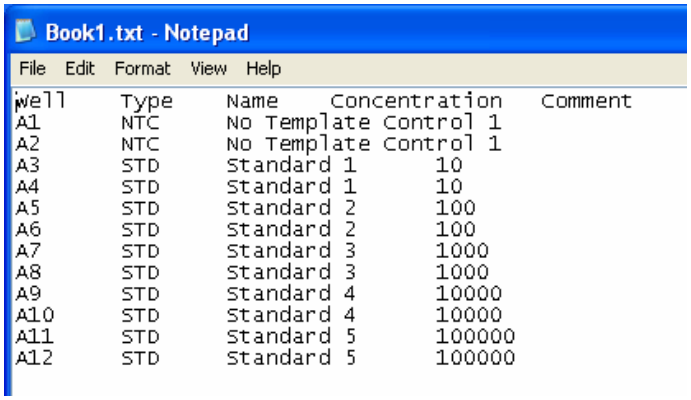
- To import from Excel into the Plate layout, select the cells to be copied and press **Ctrl+C** or select **Copy**. Note that the headings (consisting of Well, Type, Name, Concentration and Comment) also need to be copied for the data to considered valid while importing.
- Go to the Plate Layout, select the Title bar of the Well Information table and press **Ctrl+V** to import the data.



Sample information can also be directly imported from a .txt file.

- On the Menu bar click on **File** then **Import from file…**. Browse the file directories to find the correct file to import.

The .txt file must be in the format shown below for the data to be considered valid.

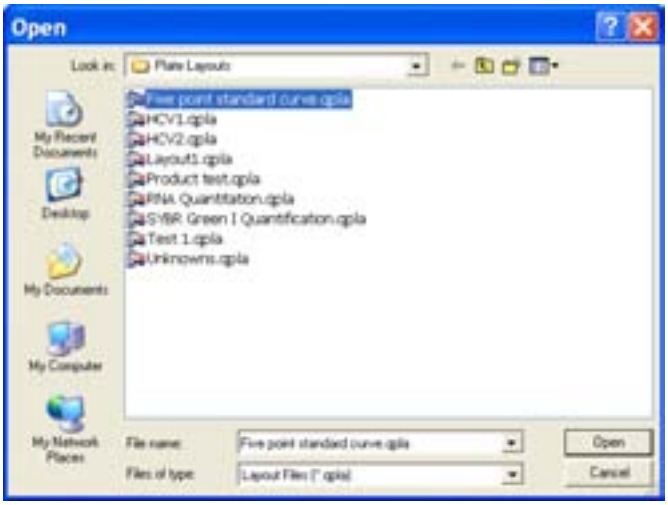


- To export Well Information to Excel either click on the Menu bar or right mouse click on the Well Information table and select **Export to clipboard**. Open up Excel and click on **Paste**.

3.4.4.9 Browse for an existing plate layout

The user can browse for an existing plate layout file by clicking on the **Browse** button in the Plate layout pane of the Experiment Editor.

This will open up the Plate Layout library folder and display any existing templates (.qpla files).



- Highlight a file and click **Open** to open up the plate layout file in Experiment Editor. The template is ready for use.

3.4.4.10 Edit a plate layout

A plate layout already present in the Experiment Editor can be edited simply and easily by clicking the **Edit** button in the Plate Layout pane of the Experiment Editor. Clicking on **Edit** takes the user back into the Plate Layout Editor and allows any of the plate layout settings to be changed either before or after the run.

- Click **Done** to return to the Experiment Editor and the edited plate layout will appear in the Plate Layout pane.

3.4.4.11 Create a new plate layout from the Home page

Clicking on **Create a New Plate Layout** from the Home Page provides a quick link through to the Plate Layout Editor. Define the plate layout for each sample type and edit the well information as detailed in section 3.4.4.

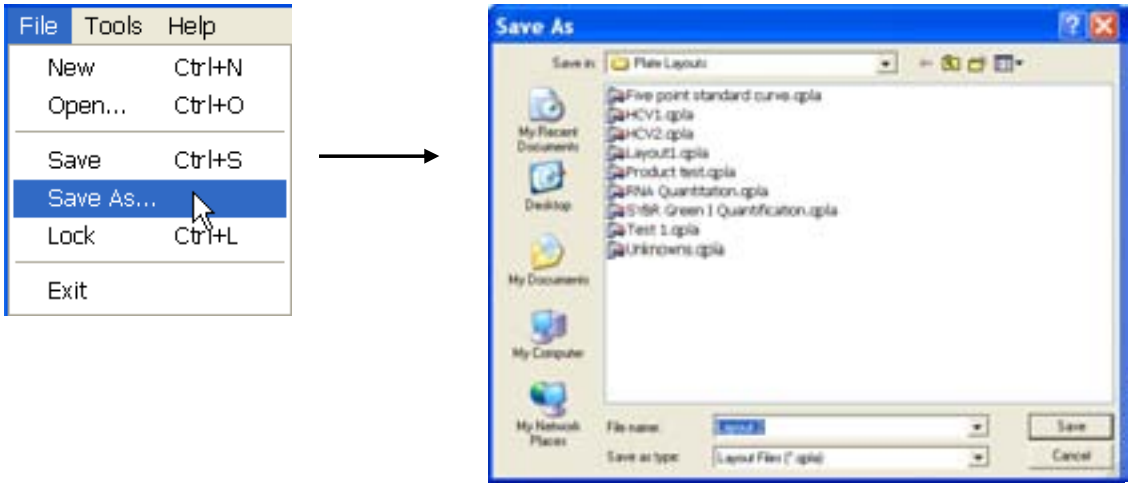
3.4.4.12 Saving a plate layout to the library folder

The plate layout file can be saved at any point during the setup using typical Windows commands.

However, if the padlock icon in the top-left of the Editor screen is locked, the current file in use is designated **read only**. This provides a useful tool to protect against the accidental over-writing of files meaning that this file would have to be saved under a different file name.

This function can be turned off by simply clicking the padlock icon or by choosing the **File** option in the menu bar and clicking on **Lock**.

- On the menu bar in Plate Layout Editor, select **File** and then **Save As…**



IMPORTANT: Certain characters must not be used in the file name otherwise it may become corrupted and you may not be able to open the Results file. These characters are: < > & ‘ “

- Change the file name as required. If an existing name is selected then a warning message will appear asking if the file should be over-written. The file (.qpla format) will automatically be saved to the directory My Documents\Quansoft\Plate Layouts. Change the destination by browsing the file directories shown in the **Save in:** drop-down menu.

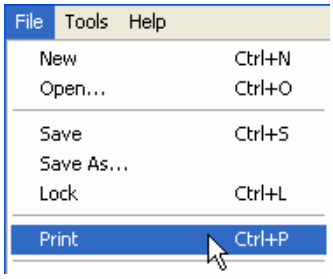
The library files can be accessed by browsing within the Plate Layout Editor as shown in section 3.4.4.9 or accessing the library folders from the quick links on the navigation bar of the Home page (detailed in section 3.3.3).

The destination of all Quansoft files can be changed using the **Tools** function located on the menu bar on the Quansoft Home page. See section 3.4.3.10 for further information.

3.4.4.13 Print a plate layout and well information

The plate layout and well information table can be printed; this can be useful for setting up the experiment in the laboratory and allows the user to keep a record for their notebook.

- On the menu bar in the Plate Layout Editor, select **File** and then **Print**.

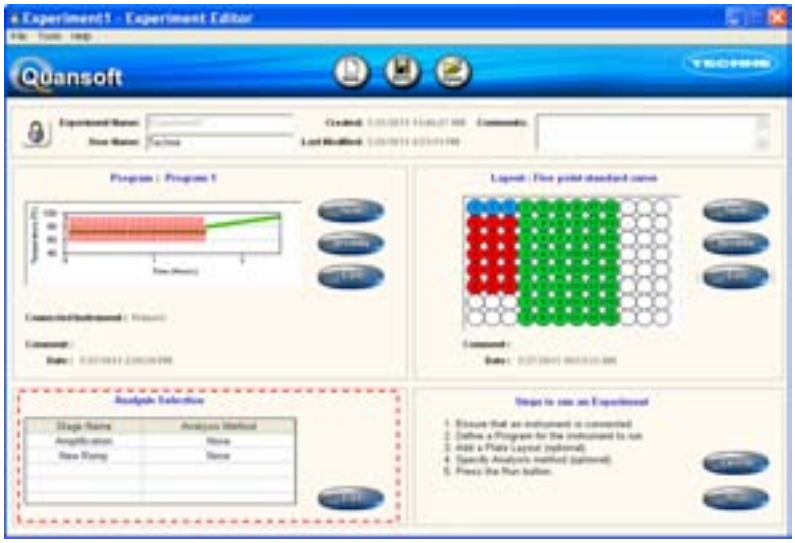


The plate layout is printed at the top of the page followed by the well information table.

3.4.5 Defining the analysis method

3.4.5.1 Selecting an analysis method

The Analysis Selection box in the Experiment Editor (also found in the Results Editor after the run has finished) allows the user to define the method of analysis to be applied to readings gathered during the PCR program.

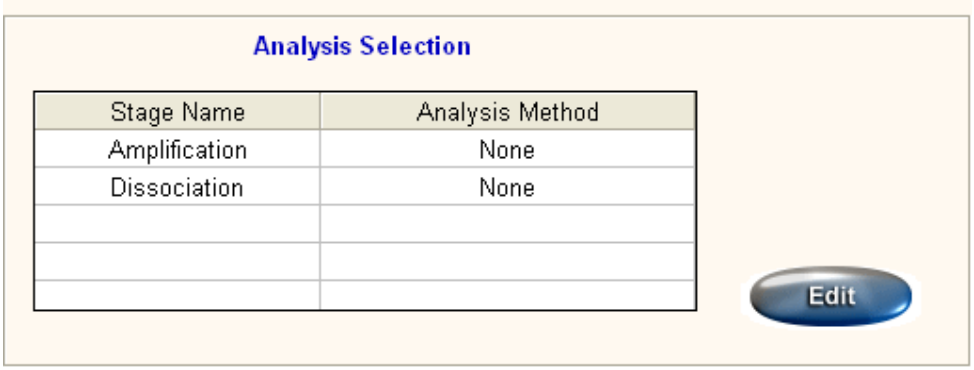


- **Analysis Selection box:** Displays the stage name as assigned in the program setup and any analysis method setup. Only those stages that have been assigned with reads (set in the Program Editor as described in section 3.4.3.5) are displayed since stages without reads have no data to analyse.
- **Analysis wizards:** Analysis can either be performed automatically using a series of default settings or set and edited using the intuitive wizard function. This will lead the user through each stage of the analysis setup.

Highlighting a stage name and pressing the **Edit** button will launch the Analysis Wizard and allow a method to be assigned for that stage.

3.4.5.2 Assign the analysis method

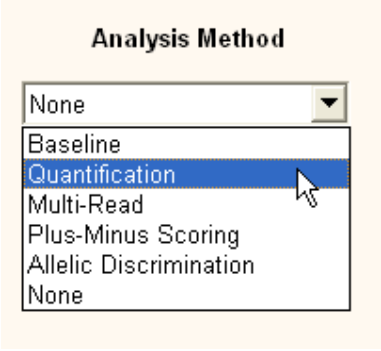
Analysis methods can be set either before the run, or defined and/or modified after the run in the Results Editor (section 3.7) using the parameters (**PAR**) functions.



- Double-click on a stage name, or highlight a stage name and press the **Edit** button to launch the Analysis Wizard Selection screen.

- **Analysis Method:** The drop-down menu allows an analysis method to be selected.
- **Dye name:** The name of the dyes selected in the program setup will be displayed.
- **Dye Usage:** Assign a Dye Usage from the list in the drop-down menu. There will be one dye usage box for each read present in the selection stage.
- **Cancel:** Aborts the procedure and takes the user back to the Experiment Editor.
- **Finish:** Accepts all the default analysis settings for the analysis method chosen and closes the Wizard.
- **Reset defaults:** Pressing this button returns all analysis parameter settings to the default.
- **Back/Next:** Allows the user to move between screens in the Analysis Wizard.

The Analysis Method drop-down menu lists analysis types appropriate to the selected stage. These will be dependent on the number of reads and the number of cycles programmed into the run (see **Choosing an analysis method** in section 4.2)



PrimeQ supports the following analysis methods:

- No analysis method defined (None)
- Baseline correction
- Quantification, including:
    a. Absolute quantification
    b. Relative quantification
    c. Relative quantification cycles (Cq)

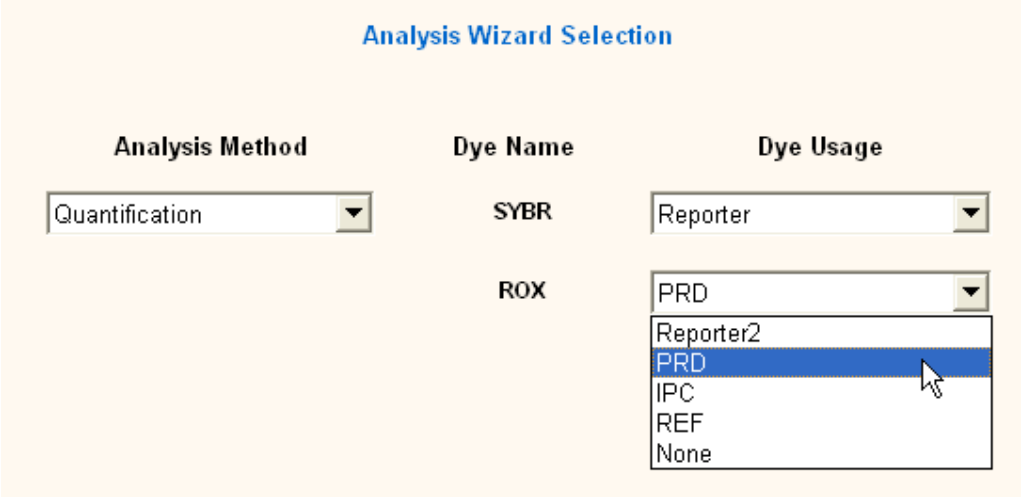
- Dissociation curve
- Plus/minus scoring
- Allelic discrimination
- Multi-read

See Chapter 4 for a specific discussion of each analysis method in terms of requirements, setup and what each approach can reveal about the experimental data.

3.4.5.3 Assigning a dye usage

As there may be more than one dye reading in a single stage, the role of each dye needs to be assigned. To do this, the Analysis Wizard Selection box has a drop-down menu containing a list of definitions for each. All definitions are treated as reporters apart from the passive reference dye (PRD). When the analysis method is undefined or set to 'none' then the dye usage selection boxes are disabled (greyed-out).

It is important to note that if an instrument is not connected and the experiment is not a template from a previous run then dyes cannot be assigned until an instrument is connected.

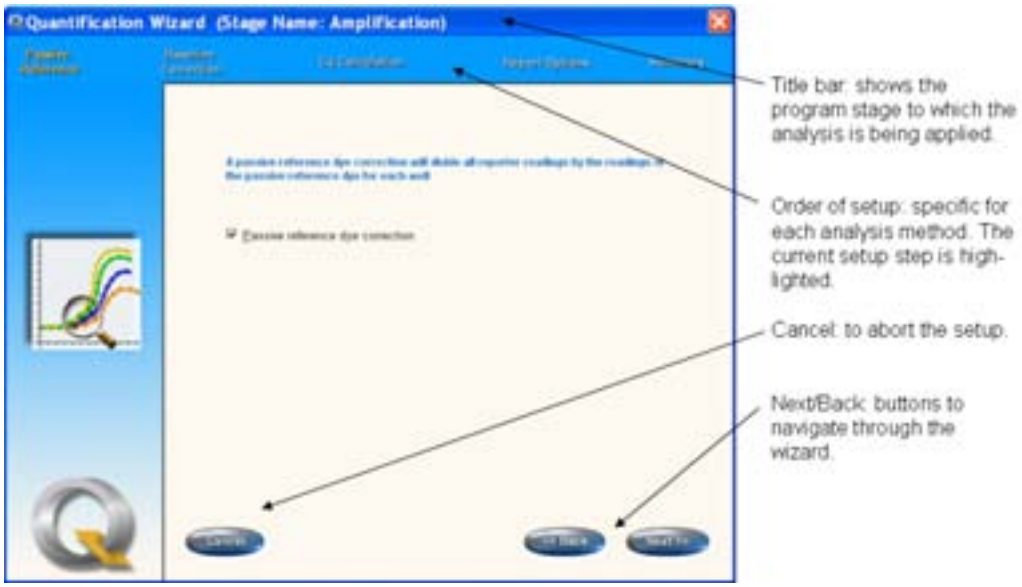


- Click **Next** to move through to the Analysis Wizard specific to the analysis method chosen.

3.4.5.4 Analysis wizards

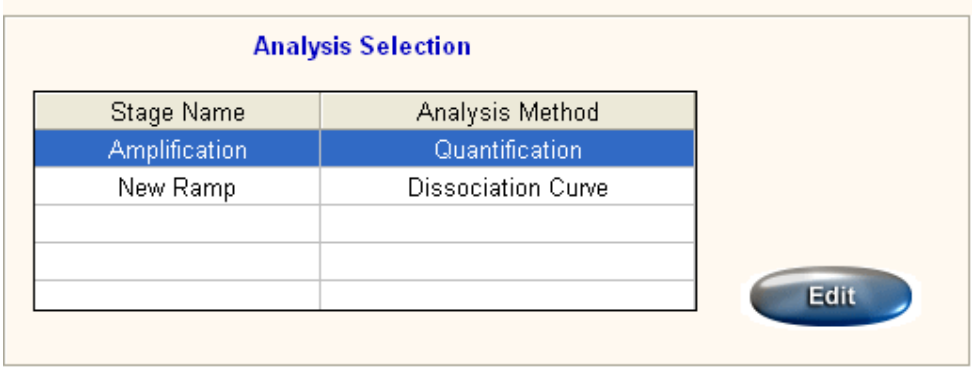
Clicking **Next** from the Analysis Wizard Selection box branches the Analysis Wizard into the individual wizards tailored to the particular method of analysis chosen. The parameters displayed will vary according to the analysis method selected and these are discussed in detail in Chapter 4. In general the Analysis Wizard will lead the user through the setup procedure as follows:

1. **Open the Analysis Wizard:**
   - In the Analysis Selection box in the Experiment Editor, highlight the stage name and click **Edit**. The Analysis Wizard Selection screen will appear.
   - Choose the type of analysis required from the drop-down menu and assign a use for each dye.
   - Click **Next** to open the selected Wizard. The Wizard screens have the following format:



2. **Passive reference dye (PRD) correction:**
   - Check the box if this correction method is required (only available if a PRD was selected in the dye usage table). The purpose of the PRD is to normalize the reporter fluorescence and make well-to-well comparisons more accurate. The readings are normalized by dividing the fluorescence of the reporter in each well by that of the PRD. This correction method is applicable to all the analysis types available on PrimeQ (see section 4.4 for more details).
   - Click **Next**.
3. **Baseline correction method:**
   - This contains a number of options and allows the user to adjust the data for any background fluorescence. The correction uses the fluorescence readings in the early cycles of the PCR while fluorescence levels are low, averages out the early noise and subtracts it from subsequent readings. As with PRD correction, baseline correction can be useful for correcting the data prior to performing an analysis and so helping to increase the accuracy of the assay. See Chapter 4 for more details.
   - Click **Next**.
4. **Set the analysis parameters:**
   - Select suitable parameters for the analysis type chosen e.g. Cq calculation method, which reads to use for end-point scoring etc. This screen helps the user decide how to choose the parameters and/or set thresholds accordingly.
5. **Report options:**
   - Clicking **Next** leads through to a window that provides options for choosing which graphs and tables appear in the report. Separate analysis methods and their report options are discussed in Chapter 4. The user can choose to select the option to run straight through to report – see section 3.7.6.3 for more information.
6. **Summary:**
   - Clicking **Next** takes the user through to a page summarizing the settings. Change any details using the **Back** function.
   - Click **Finish** to complete the setup and return to the Experiment Editor.

Once the Analysis Wizard has completed, the Experiment Editor pane is updated with the selected analysis method.



3.4.6 Saving an experiment to the library

The experiment is now complete with a program file, plate layout and analysis method all defined. While the program and plate layout can be saved as separate files (shown in 3.4.3.10 and 3.4.4.12), the experiment can also be saved as a consolidated .qexp file at any point during the setup in the Experiment Editor.

If the padlock icon in the top-left of the Experiment Editor screen is locked, the current file in use is designated **read only**. This provides a useful tool to protect against the accidental over-writing of files meaning that this file would have to be saved under a different file name.

This function can be turned off by simply clicking the padlock icon or by choosing the **File** option in the menu bar and clicking on **Lock**.

IMPORTANT: Certain characters must not be used in the file name otherwise it may become corrupted and you may not be able to open the Results file. These characters are: < > & ‘ “

- On the menu bar in the Experiment Editor, select **File** and then **Save As…**
- Change the file name as required. If an existing name is selected and the padlock icon is closed, then a warning message will appear asking if the file should be over-written.

The **.qexp** file will automatically be saved to the directory My Documents\Quansoft\Experiments. Change the destination by browsing the file directories shown in the **Save in:** drop-down menu. The library files can be accessed by the quick links on the navigation bar of the Home page (detailed in section 3.3.3) or by clicking on the **Run an Experiment** on the Home page, which will automatically open the Experiment Library folder for browsing.

The destination of all Quansoft files can be changed using the Tools function located on the menu bar on the Quansoft Home Page. See section 3.4.3.10 for further information.

3.4.6.1 Editing an experiment

Previous experiments saved in the Experiments folder can be edited for new runs.

- Access the Experiment library by clicking on **Run an Experiment** on the Home Page or using the Experiment shortcut icon on the navigation bar.

Files can be previewed by clicking on the file name and the contents will be displayed in the lower pane of the screen.

- Double-click on an experiment file to open it in the Experiment Editor. Files are located in C:\My Documents\ Quansoft\ Experiments.

The experiment opens in the Experiment Editor window.

Each element can be edited in the same way as if creating a blank plate layout, program or setting up a new analysis.

- Click **Edit** in the program or plate layout pane of the Experiment Editor and the appropriate editor will open.
- Change settings in any step/stage, alter readings and so forth or delete the current template and import another from the library files.
- Analysis: Either double-click the name or highlight and press **Edit**. Make any changes in the same way as when setting up the analysis. Change the title of experiment if you do not want to over-write the original file and add any comments if required.

3.5 Running an experiment

3.5.1 Starting the run

3.5.1.1 Preparing the system

- Turn on the power to PrimeQ using the power switch at the rear of the instrument. An idle screen will appear on the instrument's LCD display.

- Turn on the PC and log in under a user name.
- Double-click the **Quansoft** icon located on the PC desktop to open the software.
- If the instrument has not been used previously, the filter cartridges will need to be installed (section 1.16).
- Prepare and insert the sample plate (as described in section 1.19).

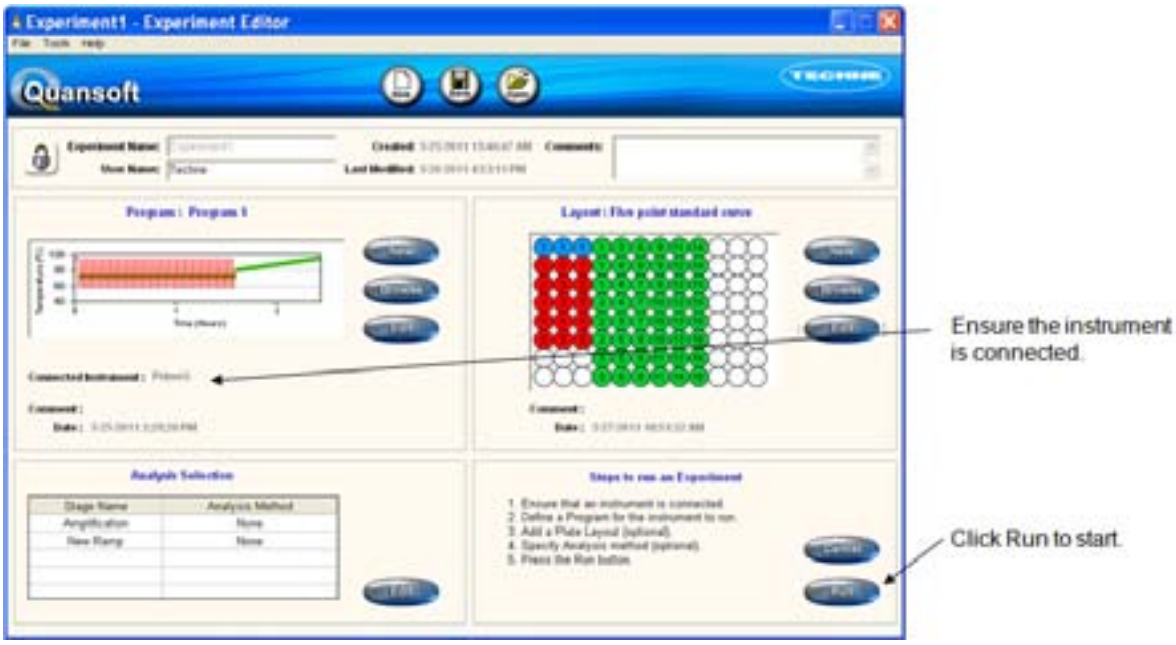
3.5.1.2 Opening the experiment file

Open up the experiment of choice in the Experiment Editor using one of the following options:

- **Set up a new experiment:** Click on **Create a New Experiment** from the Home Page and set up a new experiment as detailed in section 3.4.2.
- **Run an existing experiment:** Choose **Run an Experiment** from the Home Page and browse the experiment library files (.qexp). Edit any element of an experiment or browse for separate program (.qprg) or plate layout (.qpla) library files.
- **Use an experiment from a previous run**: An experiment file can be created from a results file of a previous run by opening a results file in the Results Library (accessible from the navigator bar of the Quansoft Home page) and selecting **Save As Experiment File** from the **File** menu.

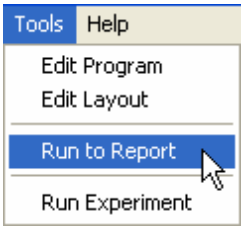
The most important element of the experiment to be considered pre-run is the **program** as thermal cycling parameters and read points must be defined to acquire the data. The plate layout and analysis methods can both be set up or edited post-run.

3.5.1.3 Running an experiment



3.5.1.4 Run to report

Unless otherwise informed, the system will wait for an acknowledgment from the user that the run has ended before proceeding on to analysis and report generation. If Quansoft is required to run straight through to report generation with no prompt or user intervention then select the **Run to Report** option found under **Tools** on the Experiment Editor menu bar.



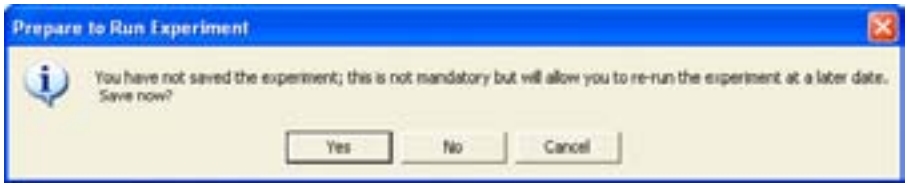
Note: running to report is only possible where the analysis type can use the default parameters or user-defined settings and does not require user intervention.

3.5.1.5 Starting the run

- Click on the **Run** button in the bottom-right corner of the Experiment Editor window. A message will appear asking for the action to be confirmed:

- Click **Yes** to confirm.

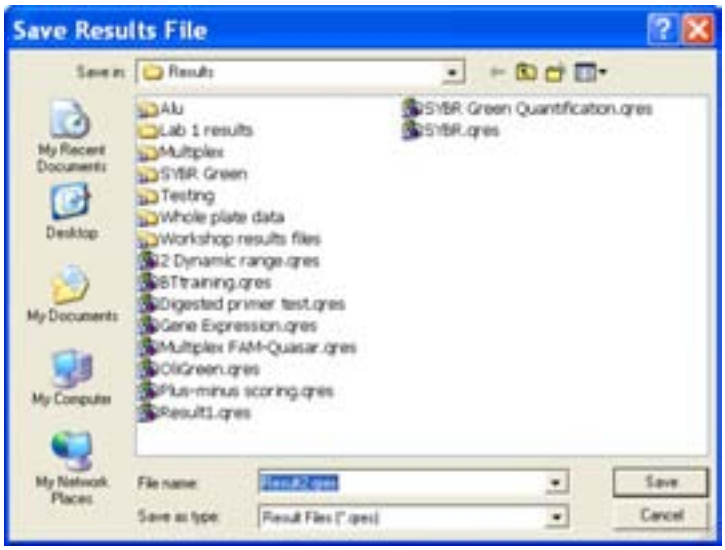
If the experiment has not already been saved or a previously run experiment is being used and has been edited, a message will appear asking if the experiment should be saved:



- Save to the Experiment library folder if required (see section 3.4.6). Note that saving will over-write the previous version of the experiment.

IMPORTANT: Certain characters must not be used in the file name otherwise it may become corrupted and you may not be able to open the Results file. These characters are: < > & ‘ “

A box will now appear asking to save the results in the Results library.



- Change the file name of the Results file if required. The file will automatically be saved to the directory My Documents\Quansoft\Results but the destination can be changed by browsing the file directory shown in the **Save in:** drop-down menu. A final message will appear asking whether to start the run.

- Click **OK** to confirm and the run will initiate

3.5.1.6 Warning messages

The user will be alerted if:

- The plate drawer has not been accessed (an indication that a plate has not been loaded).
- The lid of the unit is open.
- The plate drawer is open.

A prompt will appear requiring the user to confirm that the relevant action has been taken i.e. close the lid/drawer or load the plate.

3.5.1.7 Additional information

If the instrument was not connected when the experiment was created, the correct cartridges will need to be selected for each read. To select the cartridges, go into the Program Editor from the Experiment Editor and assign cartridges to each read. Alternately when the run button is pressed the following error window will appear:

Selecting **OK** will take the user to the correct window where all of the reads can be assigned a filter cartridge.

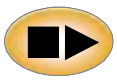
3.5.2 Monitoring the run

3.5.2.1 From the instrument

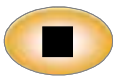
Progress and current status is reported via the display screen on the front of the instrument.

- **Cycle cc/mm:** Reflects the current cycle number out of the total number for that stage eg. 7/33 is the 7th cycle out of 33. This re-sets after each stage.
- **xxhyy:** Indicates the calculated time remaining for the entire PCR run where xx is hours and yy is minutes.
- **Sample:** Indicates the current block temperature.
- **Status:** Indicates the current thermal status e.g. ramping or holding.

During a run, pressing the keypad will have the following effect:



Attempts to pause run. The screen will ask for confirmation of the pause command.

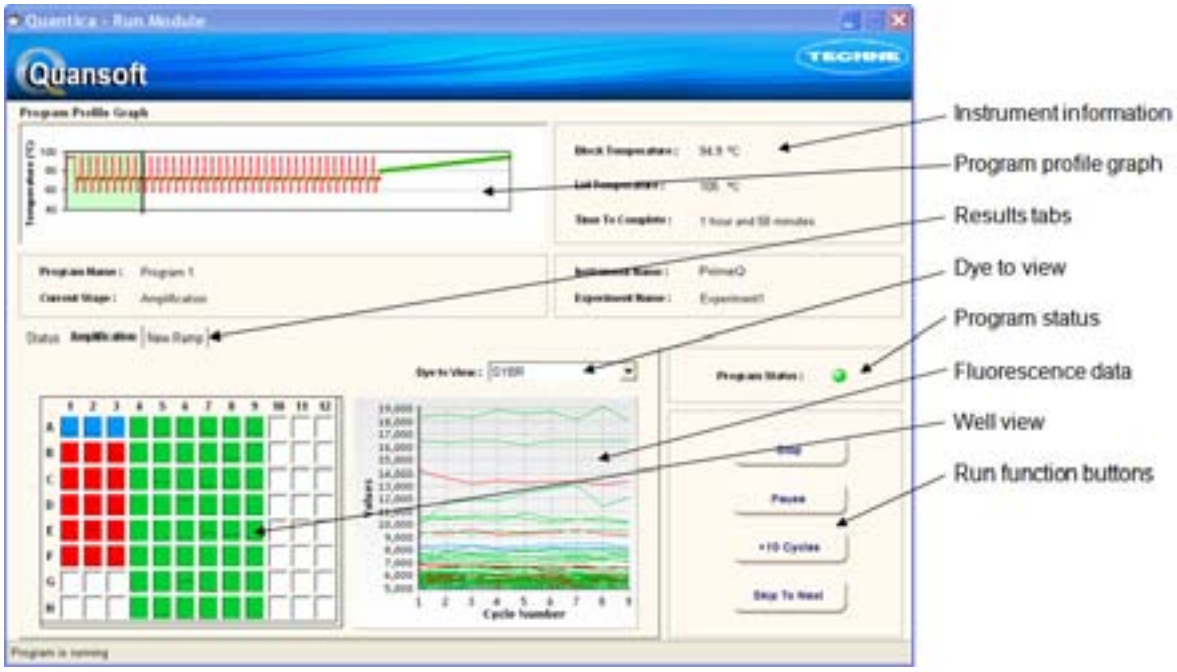


Attempts to stop run. The screen will ask for confirmation of the stop command.

Displays the **Run Information** screen. This displays details of the instrument, experiment and stage names.

3.5.2.2 From the Run Screen

As the PCR is in progress, the Run Screen displays the current status of the program in real time. Fluorescence data is shown on a per-well basis with the different stages of the run (which have readings) being displayed as separate tabs. The user can select an individual well to show the fluorescence data for that well only. The readings are time-stamped and saved in the results data. If **Run to report** was chosen from the menu bar, on completion of the run the software will automatically analyse the data and produce a report based on the user-defined settings.



- **Instrument information:** Displays various details identifying the instrument, experiment and program. The block temperature, lid temperature and time to completion are also shown.
- **Program profile graph:** Contains a profile of the PCR program depicting all cycles in the run. The red line shows the temperature profile while the coloured circles are the dye reading points (more than one dye is represented by different colours as defined during the program setup). A vertical line provides the user with a visual representation of how far the run has progressed. The program name and the stage the program is currently in are both displayed at the bottom.
- **Results tabs:** Each stage in the program that has been assigned with a read has a tab labelled with the stage name given during the program setup. The current stage is located uppermost but the user can click between tabs while the run is in progress to view results for other stages.
- **Dye to view:** Where multiple readings are being taken, change the reporter being displayed using the drop-down menu. Only dyes assigned in the setup will be displayed so if only a single reporter has been assigned, this option will be disabled.
- **Status lights:** Indicate the instrument status

  Instrument stopped or faulty

  Instrument paused

  Instrument run in progress

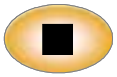
- **Fluorescence graph:** Data for a particular stage will be displayed in the graph. Choose to display readings for the entire plate or just for selected wells. Change the dye being viewed using the **Dye to View** option.
- **Well view:** A fluorescence curve is displayed in each well as the PCR progresses. Highlight a well(s) with the mouse to display the fluorescence data for that well(s) in the adjacent graph. To return to viewing all filled wells, click just outside the top left-hand corner of the plate view. Click again to leave all wells viewed but deselected (highlighted).
- **Stop/Pause:** These commands can be activated from either the PC or the instrument (provided the instrument buttons were not disabled in the Program setup). If the stop button is pressed then the user is queried as to whether the run should be stopped. If confirmed, the instrument is sent an abort command and the software moves into the analysis with whatever readings have been collected up to that point.
- **+10 Cycles:** Adds 10 cycles to the current stage and is useful if the PCR is progressing slower than expected.
- **Skip to Next:** Skips the remainder of the current stage and starts the next stage. The program profile updates accordingly. Note: if skipping to a Ramp Read stage it is recommended that the skip occurs at the lowest temperature step in the previous cycle.

3.5.3 Stopping or pausing a run

3.5.3.1 From the instrument

The run can be stopped from the instrument LCD control buttons as long as they were not disabled during program setup.

Press to pause the run. The screen will ask for confirmation of the pause command.



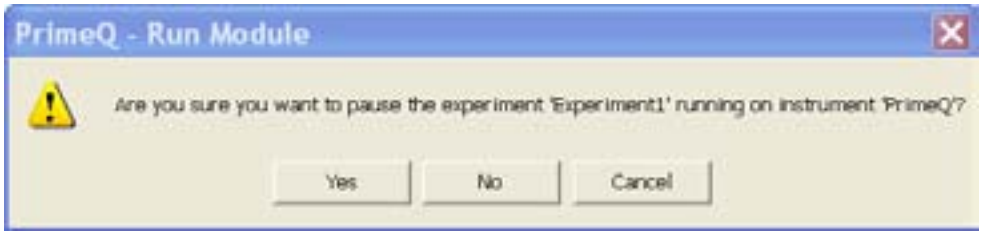
Press to stop. The screen will ask for confirmation of the stop command.

Information only.

3.5.3.2 From the Run Screen

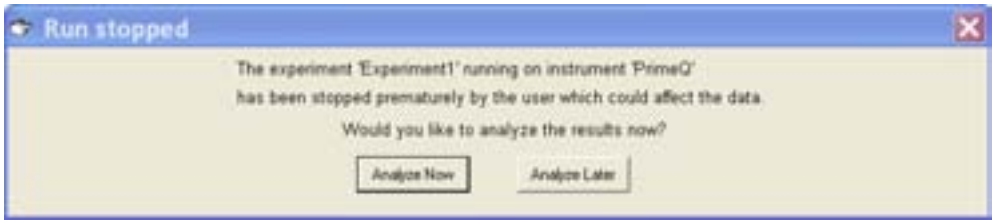
The run can be paused or stopped from the Run Screen within Quansoft.

- Click the **Stop** or **Pause** buttons and confirm the command when prompted.



When a run is paused, the temperature will be held at whatever point the pause was confirmed.

If a run is stopped prematurely, the software does not progress to the analysis screen but alerts the user as to what has occurred via an information dialogue box.



3.6 LED intensity settings

Please note the following:

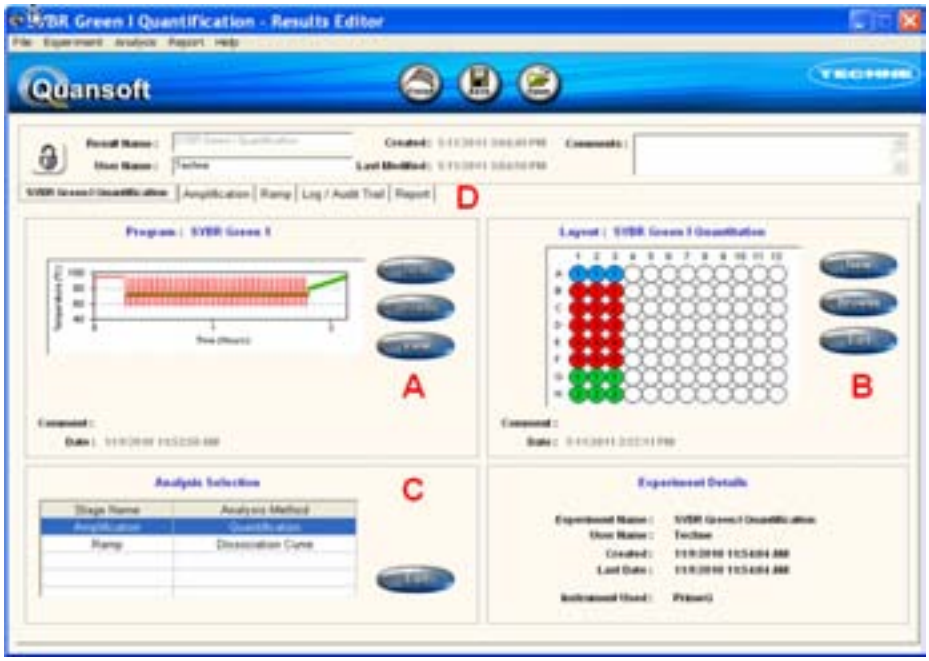
- If the fluorescent counts on the raw data plot of the Run Screen or Results Editor are above 100,000 counts using the Medium LED setting, it is recommended that the Low setting be used for the assay instead.
- Similarly, if there are less than 1,000 counts on the Medium setting, use the High LED setting instead.

3.7 Results Editor

3.7.1 Post-run analysis main screen

At the end of a run, if an analysis method was assigned to a stage with a read, the analysed readings (calculated using either the software defaults or parameters set by the user prior to the run) will be presented in the Results Editor. If no analysis method was setup, only the raw fluorescence data will be displayed.

The appearance of the main screen closely resembles that of the Experiment Editor, displaying the program, plate layout and analysis information in their respective panes and allows the user to change the plate layout and analysis parameters post-run. The main screen, as shown, is always the first, left-hand tab.



A. **Program pane:** Allows the user to view the program settings but not to edit the program (since the program has completed). As such, the New and Browse functions are greyed-out.

- Click **View** to open the Program within the Program Editor. The program will appear as it did on setup, the only difference being that the user cannot edit or change any settings.

B. **Plate layout pane:** Appears as it does in the Experiment Editor and has the same functionality in terms of editing the layout, browsing for a template from the library files or designing a layout from new (section 3.4).

C. **Analysis Selection:** Appears as it does in the Experiment Editor and also has the same functionality.

- Click **Edit** to enter the Analysis Wizard to view the settings or to change the method of analysis. The results tab appropriate to the stage will then be updated accordingly. The user can change any of the parameters and re-analyse the results as many times as desired as long as the experimental design supports a particular analysis method.

D. **Results tabs:** The results for each of the program stages assigned a read can be viewed by clicking on the appropriate tab. The results for each stage are analysed according to the defined analysis parameters as discussed in Chapter 4.

3.7.2 Viewing the results of a run

Click on a results tab on the Results Editor main screen to view the results for a particular stage. The stage name tab, called here Amplification, brings up the results relevant to that stage analysed according to the user-defined settings.

A. **User and experiment ID:** A user name can be entered and comments specific to the experiment can be added in the **Comments** box.

B. **Results tabs:** To the left of the main screen tab are a series of Results Editor tabs. The uppermost (active) tab displays the Result Editor page as shown. The tabs then list the stages in the order that they ran. Clicking on the tabs will display the results and graphs relevant to that particular stage. It is also possible to move between stages from the Results Editor menu bar.

   - From the **Analysis** option, click on **Show Stage** and then the name of the stage to be displayed.
   - Also found on the results tabs is the **Log/Audit Trail** tab, which provides details of the run useful for GLP purposes (see section 3.7.5), while the Report tab displays the information sent to the PrimeQ report (see section 3.7.6).

C. **Combine replicates:** Clicking this button combines replicate readings to provide an average for a sample. The button will then change to **Show all Samples** – click to revert to the expanded data.

D. **Dye to View:** Where multiple readings have been taken, the reporter being displayed in the graphs and results table can be changed using the drop-down menu. This option is disabled if only one reporter is being used. Note that a PRD is not regarded as a reporter so if one of the dyes was selected as a PRD in the Analysis Wizard, it will not be listed as available for viewing.

E. **Plate layout:** The plate layout is always displayed and shows the colour-coded well types (standards, unknowns etc.) as defined in the Plate Layout Editor.

   - Fluorescent readings appear on a per-well basis with a small graph displayed inside each sample well.
   - Wells can be selected as in Microsoft® Excel (click, ctrl-click, shift-click, drag, select column/row/all), with only the selected wells appearing on the relevant analysis graphs.

- Hover the mouse over a particular well for information about the sample type given in a tool-tip.
- Selected wells are persistent between dyes.
- Click outside the well plate on the top left hand corner for all filled wells to be viewed; clicking again leaves all wells viewed but not selected (highlighted).
- **Right mouse** brings up a menu containing **Flag wells** (or 'Unflag' if flagged already). A flagged well has a cross displayed and is removed from all calculations (appears in the results table as a strike-through). This is an important tool for selectively eliminating a well from the calculations.

F. **Results table:** Contains the calculated results for the run.

- The results table can be maximized for improved visibility.
- Use the **Combine Replicates** function to display an average of replicate readings (click again to revert).
- The calculated results will vary according to the method of analysis – see Chapter 4 for analysis-specific explanation.

G. **Graphical display of data:** The graphs displayed in the Results Editor will vary according to the analysis method chosen and the report options selected during setup (further details for each analysis method can be found in Chapter 4).

The icons found at the top-right of each graph allow the user to:


Maximize a pane – the graph will fill half the screen so aiding visibility.


Minimize a pane – will revert a maximized graph to normal size.


Close a pane – remaining panes will be automatically rearranged.

Brings up the analysis parameters relevant to the graph (see **Changing the analysis parameters** in section 3.7.4).

H. **Graph icons:** Clicking the icons at the top-left allow the user to open any graphs or results that either have not been shown automatically or that have been actively closed. While specific icons do appear for specific analysis methods (see Chapter 4), the following examples are common to all or most analysis methods.


Displays a graph of raw data showing fluorescence plotted against cycle number.


Displays a graph of the background corrected data


Displays a graph showing the block temperature


Displays the results table


Displays a graph of the standard curve (where applicable).


The PrimeQ Report can be printed simply by selecting the Print option within the Report tab or selecting the print icon.

I. **Show All Wells:** Clicking this button restores the graphical display of data from all wells if previously only one or a few wells have been highlighted. This is particularly useful if one of

the graphs has been maximized as the user does not have to return to the plate layout display to re-select the data.

J. **Finish/save icons:** The results file can be saved at any point during the setup using typical Windows commands.

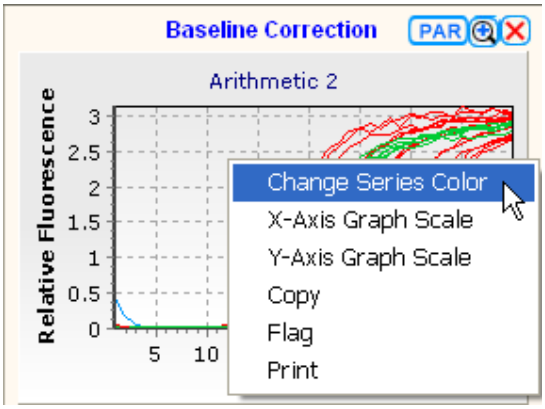
However, if the padlock icon in the top-left of the Results Editor screen is locked, the current file in use is designated **read only**. This provides a useful tool to protect against the accidental over-writing of files meaning that if a file of the same name exists in the destination folder, this file would have to be saved under a different name. This function can be turned off by clicking on the padlock icon.

The user can also choose to save the experiment as just an experiment file i.e. minus the results. On the **File** option on the menu bar, choose **Save as Experiment File** and the data will be saved as a **.qexp** file into the default Experiment Library folder. If the padlock icon is locked then the file cannot be given the same name as an existing file in the destination folder. Assign a different name or 'unlock' the padlock by clicking the icon in the top-left of the Results Editor.

The user can finish the analysis at any time by selecting the **Finish** icon. If there have been any changes made since the results file was last saved, the user will be prompted to save the file.

3.7.3 Editing a graph

Simple changes to a graph can be made using the **right-click mouse** function while on the graph of choice. This will bring up basic options for changing the appearance of a graph.

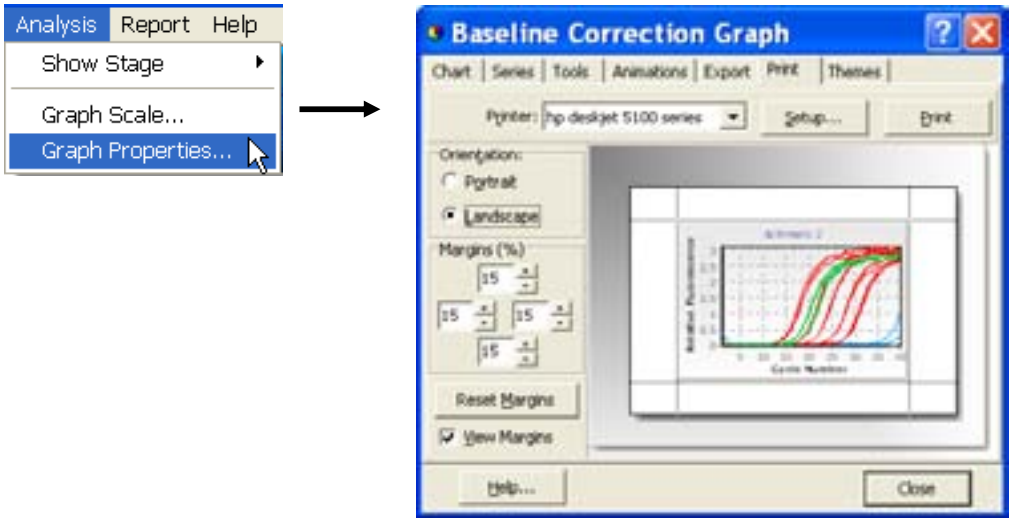


- **Change Series Colour:** A palette will appear allowing the user to change the colour of the data points.
- **Graph scale:** Clicking will display the **Format Axis** settings box. This allows the user to change the title and scale of the X- or Y-axes. Change as required and then press **Done** to finish. This option can also be accessed direct from the menu bar – click on **Analysis** and then **Graph Scale**…
- **Copy:** Copies the graph to the clipboard.
- **Flag:** Removes selected data from the graphical display and subsequent analysis.
- **Print:** Right click and select Print to print any graph.

Change the **Graph scale** as follows:

- **X- and Y-axis graph scale:** Clicking will display the **Format Axis** settings box. This allows the user to change the titles and scales of either the X- or Y-axis.

- If more sophisticated editing functions are required, access the **Graph Properties…** option from the Results Editor menu bar. Select **Analysis** and then **Graph Properties…**



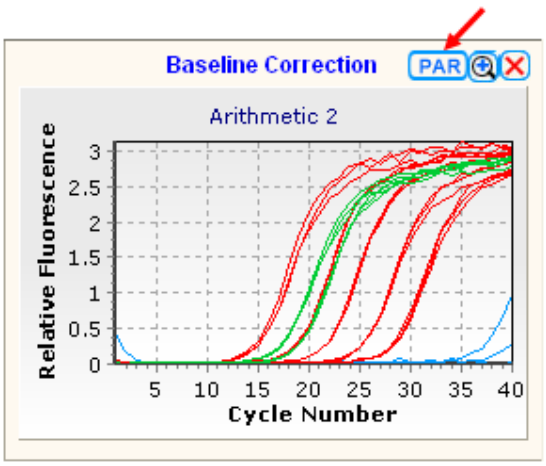
The graph editing box is equipped with many features that allow the user to edit different aspects of the graph including its appearance, the data itself or the print and export options. One of the most important features is the Export tab which enables the user to export the graphs in different file formats.

3.7.4 Changing the analysis parameters

A flexible approach to data analysis is provided with the option to re-analyse the results of a run using different analysis parameters or with a different analysis method entirely (as long as the experiment design supports the analysis method).

3.7.4.1 Edit analysis parameters

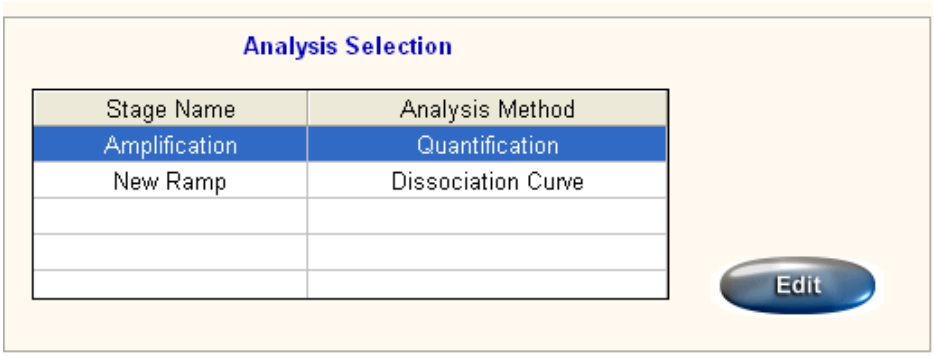
The settings for an analysis method can be viewed and changed from within the Results Editor by clicking the **PAR** button next to the graph.



Clicking this button brings up the analysis parameters relevant to the analysis being performed. The parameters can be edited with changes reflected immediately in the adjacent graph(s).

3.7.4.2 Change the analysis method

An entirely different analysis method can be chosen or existing parameters edited from the Analysis Selection box found on the Results Editor main page.



The principle works exactly the same as for pre-run setup: simply enter the Analysis Wizard by highlighting the stage name in the Analysis Selection table and click **Edit**.

3.7.5 Log/Audit trail

Clicking on this tab displays all the information about the run that may be required for GLP purposes. It is separated into an Experiment Log, Instrument Log and Audit Trail.

- **Experiment Log:** User and experiment details.
- **Instrument Log:** Details of the program such as stage name, number of cycles, temperature and ramp rate.
- **Audit:** Includes a summary of the experiment details such as the user, date, time, old/new parameters which have been selected.

Scroll through the pages using the reverse and forward arrows above the page. Print using the Print Report icon.

Go to first page/previous page.

Go to next page/go to last page.

3.7.6 Report

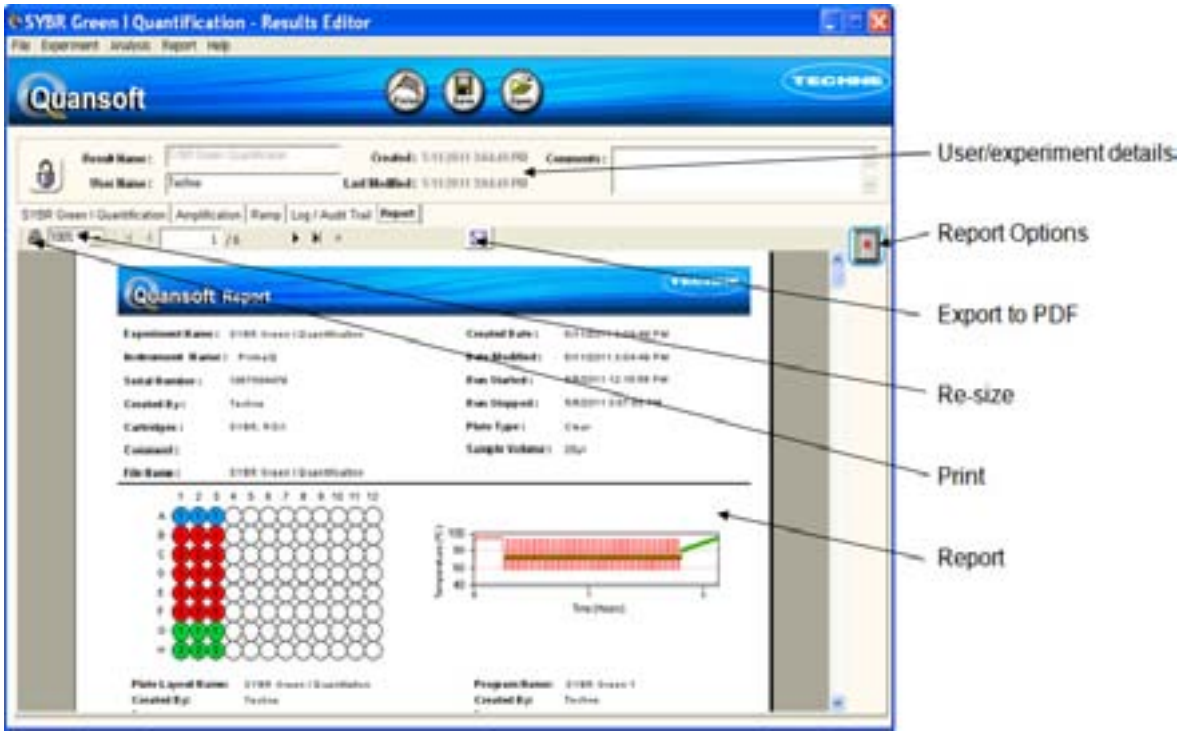
The PrimeQ Report can be viewed by clicking on the **Report** tab in the Results Editor. The results displayed will depend on the report options selected during the analysis method setup (in the **Report Options** pane of the Analysis Wizard) although default details included in the report are:

- User
- Date
- Experiment name
- Protocol name
- Instrument ID
- User-inputted comments
- Plate layout
- PCR program:
    - Temperature profile plot including where reads occurred

- Summary of filters used at each stage, emission and excitation wavelengths and user-defined name
- Integration time

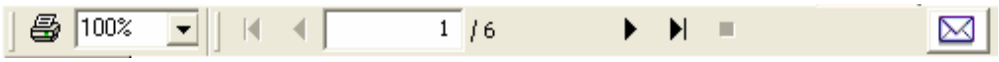
- Table of results

3.7.6.1 Report layout



3.7.6.2 Report screen function buttons

Frequently used functions are found on the **Task bar**:



	Show current page number out of total.
	Go to first page/previous page.
	Go to next page/go to last page.
	Export to .pdf.

The report options can easily be changed from within the report tab.

- Click on the **Report Options** icon to open the Report Options box as seen in the Analysis Wizard. Tabs will display the report options for each stage.

- Change any settings as appropriate and click **Done** to finish or press **Cancel** to return to the Report.

The Report Options box: change how the data is displayed using these settings.

3.7.6.3 Run to report

If this option has been chosen on setup (from the **Tools** option on the Experiment Editor menu bar), at the end of the run, the software will automatically analyse the data and run through to report based on the user-defined options during setup. If no analysis has been set then only the plate layout and fluorescence data will be shown.

3.8 Exporting and printing results

The advantage of the Results file format is that it conveniently combines all the information about an experiment together with the results data. Due to the inherent flexibility of the Quansoft platform, the user is able to either re-run the experiment unchanged or take separate elements and create or edit a new experiment.

3.8.1 Exporting

- **PrimeQ files:** Plate layouts, programs, experiments and results files are all easily transportable as electronic files – simply double-click on the file and it will open straight up into the appropriate program. If transferring multiple files then a file compression program, such as WinZip, will be useful. To export a file, simply highlight the results file, right-click with the mouse and then choose **Send to**. If WinZip is installed, the file can be sent for compression.
- **Results data:** The results of an experiment (with calculations if appropriate) can be exported to Microsoft® Excel for viewing or manipulation in a spread sheet form. In the Results Editor, choose the experiment stage for which data is to be exported by clicking on the appropriate tab. From the **File** option on the menu bar, select **Export** and a **Save As** box will appear allowing the user to browse for a destination directory. Rename if required or if the padlock function is selected (as detailed above).
- **Graphs:** Right-click with the mouse on the graph to be saved and a menu will appear. Select **Copy** and the image will be saved to the clipboard. The graph can then be pasted into other common Windows programs such as Microsoft® Word, Excel and PowerPoint etc.

  Graphs can also be edited using a plethora of options, exported and printed from the **Analysis**/**Graph Properties** option on the Results Editor menu bar - see section 3.7.3.

- **Report:** The Report can be exported to a .pdf file by clicking on the letter icon.
- **Raw data:** Raw fluorescence readings can be exported directly to Excel for manipulation by the user or other software packages.
    - Click on **Start**/**All Programs**/**Techne**/**Quansoft**/**Utilities**/**Data Extractor**.
    - Browse for the Results File name and click **Open**. Individual tabs show details of the experiment, plate layout and stages with readings.
    - Use the drop-down menu to select the readings from different dyes.
    - Click on **Copy** to copy the data to the clipboard. The data can then be pasted into Excel.

3.8.2 Printing

The PrimeQ Report can be printed simply by selecting the **Print** option within the Report tab or selecting the **Print** icon.

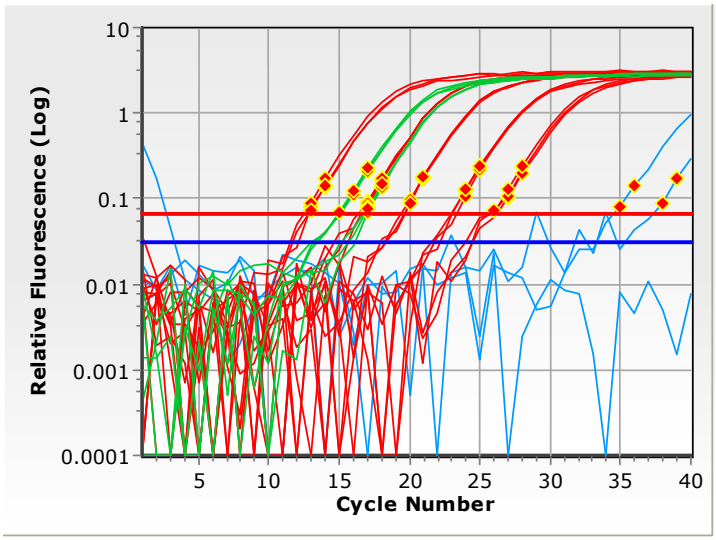
Individual graphs can be printed by right-clicking with the mouse and selecting **Print**. Alternatively they can be printed using the **Graph Properties** option accessed from the **Analysis** menu bar in the Results Editor (detailed in section 3.7.3). Various options are displayed such as copying, sending and saving in a range of formats or choose the **Print** tab to select the appropriate settings for printing.

4 Data analysis

Data analysis

About this chapter

This chapter looks in more detail at the different analysis methods available to users of PrimeQ. Following a brief introduction providing a reminder of the theory behind real-time PCR data collection, the chapter goes on to discuss each analysis in more detail including setup, applications and tips for hands-on use.

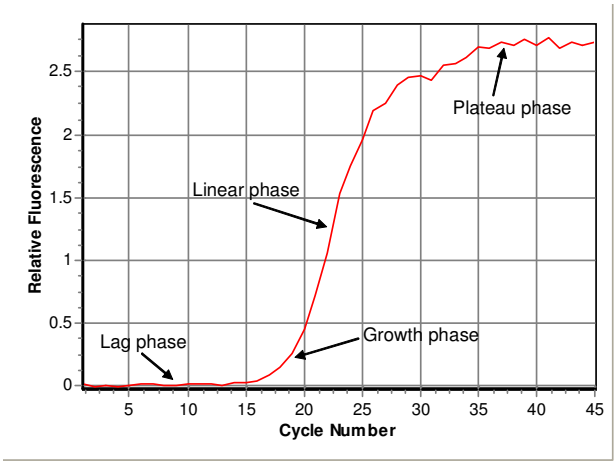


4.1 Introduction

PrimeQ can be used to determine the absolute or relative quantity of a target DNA template in a given test sample by measuring the cycle-to-cycle change in the fluorescent signal. It can also measure the simple presence or absence of a target. The fluorescent signal is taken to increase proportionately to the amount of amplified DNA and quantification is performed by comparing the fluorescence of a PCR product of unknown concentration with the fluorescence of several dilutions of an external standard. To be able to make this comparison, however, the fluorophore must be measured at a point in the amplification where the reaction efficiency can be considered comparable. This concept can be explained more clearly by looking at a typical amplification curve.

4.1.1 Amplification curve

If a PCR is plotted as fluorescence against cycle number, amplification of a PCR product would generate a curve similar to the one shown below.



The reaction curve is made up of distinct regions characteristic of PCR progression.

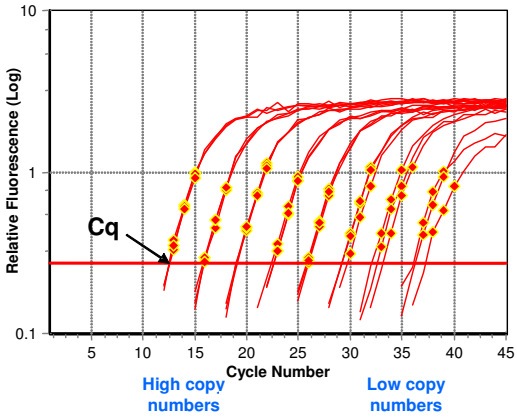
- **Lag phase (background):** Low-level fluorescence seen in the early cycles of the PCR while the target DNA copy number is still low. This can be subtracted from the readings to eliminate the level of background fluorescence. Background subtracting the data allows the user to calculate the point at which the DNA has been amplified to a level that can be detected above the noise.
- **Growth phase (exponential):** The most useful data occurs during the cycles where readings are above the noise and increasing in an exponential fashion. The phase is characterized by high and constant amplification efficiency and occurs between the first detectable increase in fluorescence and the plateau phase. If fluorescence vs. cycle number is plotted on a log scale, the plot should be a straight line.
- **Linear phase:** This phase is characterized by a steady levelling off of the amplification curve. At this point, at least one of the reaction components has fallen below a critical level and the amplification efficiency has begun to decrease (in a 100% efficient reaction, a doubling of the target should occur every cycle). As the efficiency is steadily decreasing during this phase, comparison between wells is likely to be less accurate.
- **Plateau phase:** As seen in a typical PCR curve, the linear phase eventually plateaus and stabilizes. At this point, one or more of the components is limiting the reaction and the increase in DNA amplification stops.

4.1.2 Thresholds

The threshold fluorescence is the point at which a reading is considered to be significantly above the noise. At this point, the reaction efficiency is expected to be at its highest with no limiting effects from reagent exhaustion.

The **crossing line** is a best-fit of where the reaction efficiency is at its highest and most constant for each reaction curve. The **quantification cycle**, or **Cq**, (sometimes referred to as the C_T in the literature), is the point at which an amplification curve intercepts the crossing line. Measuring fluorescence at the Cq for each well will allow the most reliable well-to-well comparison.

Plotting the Cq against the known concentration of standards will generate a standard curve that makes it possible to estimate the starting concentration of unknowns.



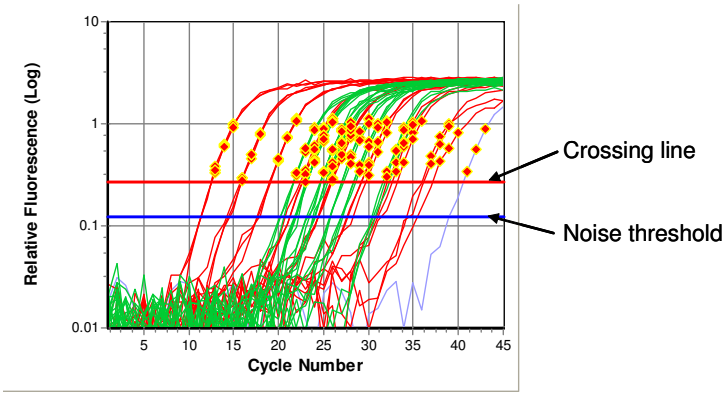
The quantification cycle (Cq) is the point where the amplification curve intercepts the crossing line.

Two approaches are available for calculating the quantification cycle on PrimeQ: fit points and first derivative maximum.

4.1.3 Fit points

This approach is the preferred approach when using low copy number templates and is based on the setting of a crossing line and noise threshold. It is the default method used by Quansoft. The noise threshold is used to define all non-informative fluorescence data – data that is typically indistinguishable from the fluorescent noise seen in the early stages of amplification.

The crossing line is set at a specified number of standard deviations (SD) above the noise line using either the default settings or those specified by the user. The default setting is 10 standard deviations above the noise, which is the average of fluorescence measured in cycles 3–10. Fluorescence is then plotted on a log scale vs. cycle number with the two cursors placed in the appropriate positions: a blue line showing the **noise threshold** and a red, the **crossing line**.



The quantification cycle is then calculated using fit points. These are a defined number of fluorescence points situated on the curve above the crossing line (default number is 2). The software uses these points in a linear regression calculation to determine the best-fit gradient for each well. The intersection of this line through the crossing line is the Cq and this will be a fractional cycle number. The Cq is calculated for each well.

Note on log scale: *The only real difference between a log and linear scale is the way the data is displayed. The lower values in the log scale are 'blown up' to make the data more readable such that the normalized values approaching zero can be seen more clearly. The small increase seen in*

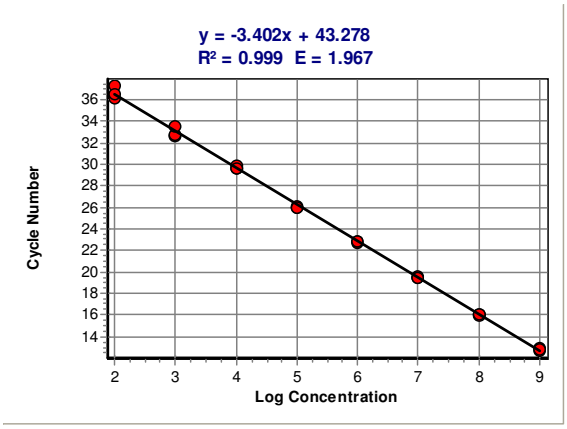
the early stages is expanded and the Cq value can be determined more accurately. Although some values may be negative when plotted on a log scale (especially if the data has been background corrected) this will not affect the accuracy of the data.

4.1.4 First derivative maximum

Simple to perform and requiring no user input, this method determines the quantification cycle by calculating the point on the reaction curve at which the rate of change in fluorescence is fastest. As this approach uses the shape of the curve for its calculations, it does not require the setting of a noise threshold or crossing line. To choose this method, simply select **First derivative maximum** in the Quantification Wizard and the software will automatically calculate the Cq value for each well by taking the first derivative of the curve and then finding the maximum. This method is better suited for high copy number templates (>1000 copies).

4.1.5 Standard curve

If standards have been defined in the plate layout, the quantification cycle for each can be plotted against the log of its concentration to produce a standard curve. Linear regression is then used to generate a straight line plot and the standard curve can then be used to calculate the starting concentration of the unknowns from their Cq values.



Plotting the Cq against log concentration of the standards generates a standard curve.

The slope of the line provides information about the efficiency (E) of the reaction whereby:

$E = 10^{-1/slope}$

A reaction of 100% efficiency would produce a value of 2 such that a doubling of an amplification product occurs each cycle. If the value is greater than 2, it suggests amplification of non-specific products, while lower than 2 suggests a component is limiting the reaction or that the reaction conditions need to be optimized.

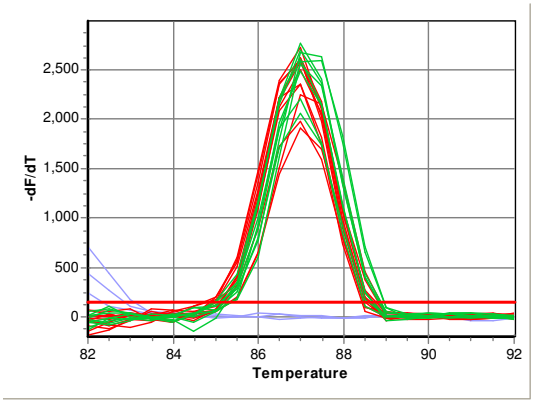
To make any comparison between samples or between two reactions in the sample well, the user has to be confident of comparing like-for-like i.e. that the reactions are being measured at comparable points in the amplification curve. This premise follows for any of the analysis methods, be it quantification, dissociation curve or allelic discrimination. This allows the user to draw true comparisons and therefore to get the most out of each assay.

4.1.6 Dissociation curve

Dissociation curve analysis can add to the information obtained from the PCR. Also known as melting curve analysis, it measures the temperature at which the DNA strands separate into single strands. This provides a measurement of the melting temperature or Tm, taken as the point at which 50% of the double stranded DNA (dsDNA) molecules are dissociated.

Using an intercalating dye such as SYBR® Green, the dsDNA is dissociated into its separate strands by a stepwise increase in temperature, with the fluorescence data collected at each temperature step. The PCR products can be seen to 'unzip' at a specific temperature (the Tm),

and as the Tm is characteristic of the GC content, length and sequence of a DNA product, it is a useful tool in product identification.



Dissociation curve showing the dissociation peaks.

At the start of an analysis, the reaction is at a low temperature such that the DNA in the reaction tube will be double-stranded and the fluorescence signal high. As the temperature slowly ramps upwards, the fluorescence will drop more rapidly as the reaction reaches the melting point and the dye can no longer intercalate. Pure, homogenous PCR products will produce a single sharply defined melting curve with a sharp peak, while primer-dimers will melt at a lower temperature with a broader peak.

Dissociation curve analysis has applications in such areas as genotyping and mutation analysis, PCR optimization and the identification and characterization of PCR products.

4.2 Choosing an analysis method

Quansoft allows the user to assign an analysis method to any stage of the PCR that has fluorescent readings. The available analysis methods are:

- **None:** When no analysis method has been set, the Results Editor will display the plate layout and a graph of raw fluorescent data.
- **Baseline correction:** Allows the user to adjust the data for background fluorescence.
- **Quantification:** Determines the amount of starting DNA:
    - **Absolute quantification**: Compares the Cqs of unknown samples against those of known standards plotted against the log of their concentrations on a standard curve.
    - **Relative quantification:** An absolute quantification method; this uses two reporter dyes and two standard curves to compare the concentration of one DNA template relative to a second template.
    - **Relative quantification cycles:** Cq values for a calibrator can be used for a relative quantification between the calibrator and all other samples in the experiment. No standards are required.
- **Dissociation curve:** Measures the temperature at which the DNA strands dissociate (i.e. the melting temperature or Tm). Since the melting temperature is characteristic of the GC content, length and sequence of a DNA product, this method is a useful tool in product identification.
- **Plus-minus scoring:** Determines the presence or absence of a PCR product – input data can either be kinetic (whereby the number of readings is >1) or end-point (one reading taken at the end of the run).
- **Allelic discrimination:** Detects single nucleotide differences – the most common assay to use is the hydrolysis probe assay using dual-labelled probes for each of the alleles of interest.
- **Multi-read:** Combines all the readings from a well and takes an average – useful in end-point analysis.

The table below provides a point summary of the valid analysis methods in terms of the number of cycles and the number of reads within a program.

Number of cycles in the stage	Number of reads in the stage	Valid analysis methods
One	One	Multi-read Plus-minus (without baseline correction or analysis method options)
Two or more	One	Baseline Quantification (without relative quantification options) Multi-read Plus/minus (all options)
Ramp read	One	Dissociation curve
One	Two or more	Multi-read Plus-minus (without baseline correction or analysis method options)

		Allelic discrimination (without baseline correction or analysis method options)
Two or more	Two or more	Baseline Quantification (all options) Multi-read Plus/minus (all options) Allelic discrimination (all options)
Ramp read	Two or more	Dissociation curve

4.2.1.1 General points

- Analysis methods can be added or changed post-run as long as the assay setup is valid for the method (see above table).
- If multiple dyes are to be corrected differently within a stage then this must be performed post-run.
- Data can be re-analysed an unlimited number of times – simply ensure any graphs or calculations of interest have been saved and change any analysis parameters or methods via the Analysis Selection box in the Results Editor. This works in the same way as when setting up parameters in the Experiment Editor. If running multiple stages with read points, data will be tabbed according to the experiment and the stage name.

4.3 Analysis method: None

The default analysis method is **None,** so analysis parameters do not need to be set.

4.3.1 Viewing the results

During the run, the real-time collection of data can be monitored in the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.

- Click on the appropriate tab to view the results of the stage.

When no analysis method has been set, the Results Editor will display the plate layout and graph of raw data.

- Highlighting an individual well displays the data for that well in the raw data graph.
- Clicking the block temperature function button brings up the block temperature graph in the lower pane.

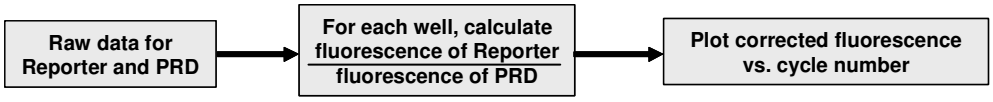
4.3.2 PrimeQ Report

The PrimeQ Report shows the plate layout and raw fluorescence data with the default run details displayed by scrolling down the screen. See section 3.7.6.

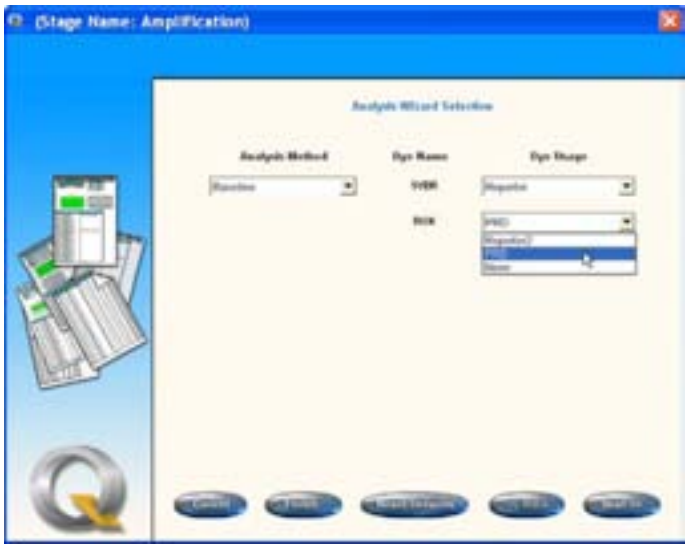
4.4 Passive reference dye (PRD) correction

The purpose of the passive reference dye (PRD) is to normalize the reporter fluorescence so that well-to-well comparisons are more accurate. The readings are normalized by dividing the fluorescence of the reporter in each well by the PRD for each well. This correction method is applicable to all the analysis types available on PrimeQ except “None”.

Setup procedure:



For the PRD correction options to be displayed, a PRD must be assigned in the **Dye Usage** box accessed from either the Experiment or Results Editor.



If a PRD is assigned, each analysis method will include options for PRD correction.

Clicking on the **PAR** button of the Raw Data graph brings up the PRD correction options.

The user can choose to display the raw data for the reporter, the PRD or the PRD corrected reporter.

The user should note that **a PRD is not regarded as a reporter**. Therefore, the dye chosen as the PRD will only appear in the **Dye to View** menu (a function that allows the user to choose which dye data is being displayed) when **None** is selected for the Analysis Method.

4.5 Analysis method: Baseline correction

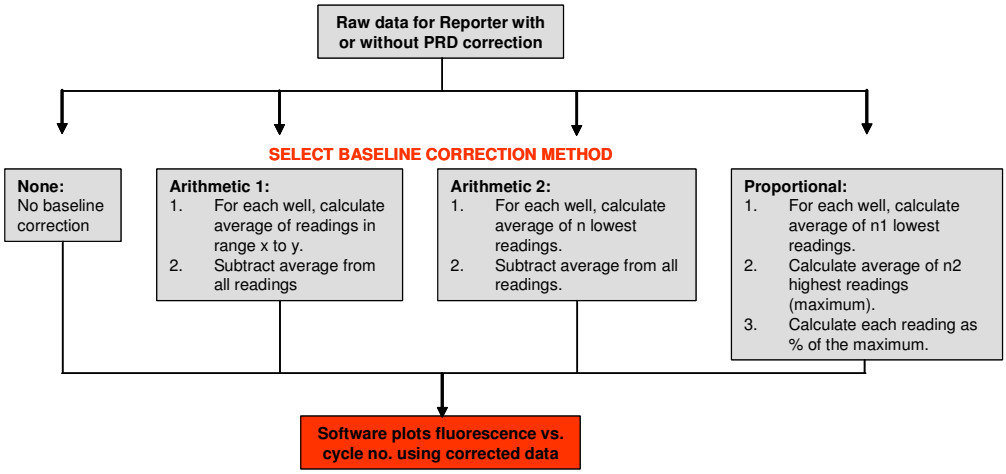
This method allows the user to adjust the data for any background fluorescence. This approach looks at the signal in the early cycles of the PCR and then averages out the early noise and subtracts it from subsequent readings. As with passive reference dye (PRD) correction detailed above, baseline correction can be useful for correcting the data prior to performing an analysis and so can help to increase the accuracy of the assay.

4.5.1 Assay requirements

- Need at least one reporter.
- Need at least two readings.
- Need a dye defined as a PRD for PRD correction options to be available.

4.5.2 Setup

The diagram below gives an overview of the different baseline correction methods.



- Select **Baseline** from the drop-down menu in the Analysis Wizard Selection box. Assign the dye usage and click **Next**. The Baseline Correction Wizard will launch.

4.5.2.1 PRD correction

If a PRD was assigned in the dye usage screen, the first screen of the wizard will ask if the data is to be PRD corrected.

4.5.2.2 Baseline correction

- Click **Next**. Options appear for baseline correction.

- **None:** No correction.
- **Arithmetic 1:** Subtracts the average of the specified range of readings from each well.
- **Arithmetic 2:** Subtracts the average of a specified number of lowest readings from each well.
- **Proportional:** The selected number of lowest readings is averaged and used to create a baseline while the selected number of highest readings is averaged to create a maximum. All remaining data is scaled between the two on a percentage basis.

The default method is **Arithmetic 2** with a default value of n = 5 (or 1 if the number of readings is less than 5). The defaults can be changed using the scrolling number boxes displayed in the lower half of the screen.

Defaults for other methods are:

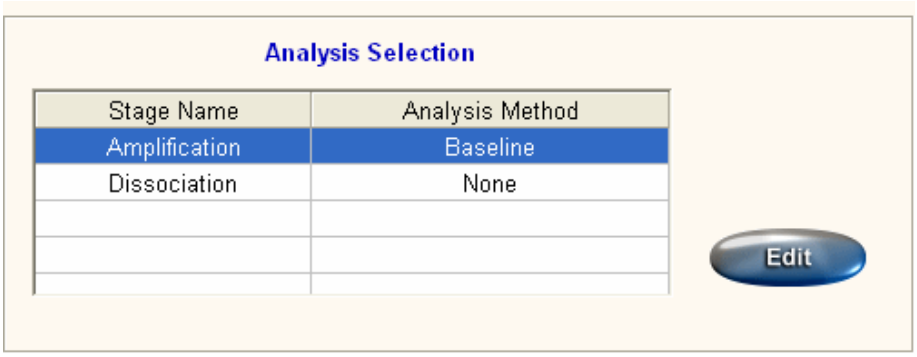
Arithmetic 1: x = 3 (or 1 if number of readings is less than 10)
y = 10 (or 2 if number of readings is less than 10)

Proportional: n1 = 5 (or 1 if number of readings is less than 8)
n2 = 3 (or 1 if number of readings is less than 8)

4.5.2.3 Report Options and Summary

- Clicking **Next** leads through to the **Report Options** screen. Baseline corrected data can be displayed in a 96-well or a graphical format – click the appropriate box to select.
- Click **Next** to view a summary of the setup.
- Click **Back** to change any settings or **Cancel** to abort the procedure.
- Click **Finish** to complete the setup.

The analysis method appears in the updated Analysis Selection box on the Experiment Editor main page (or Results Editor if post-run).



- To change any settings, simply click **Edit** to re-enter the wizard.

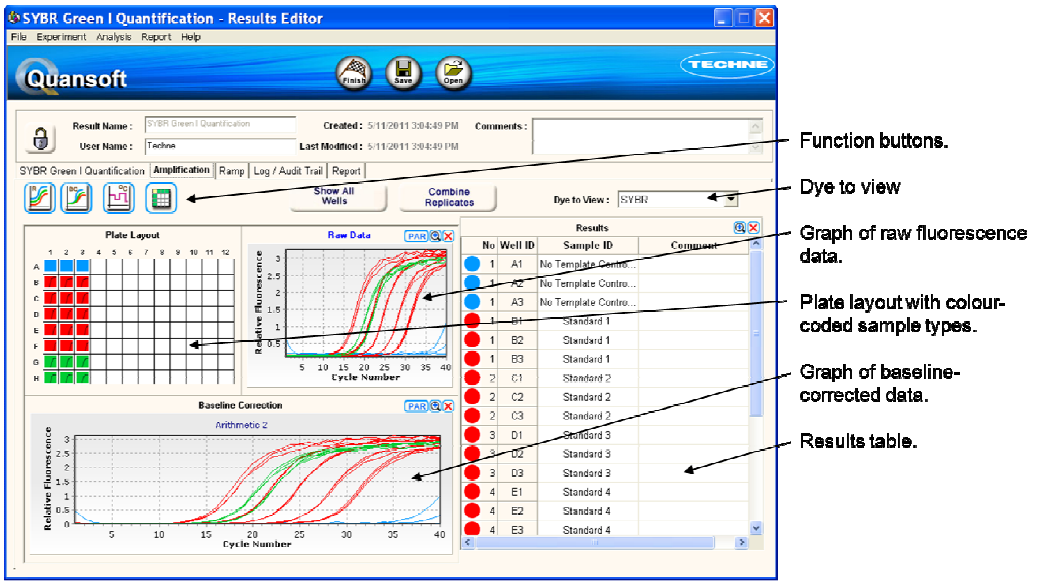
4.5.3 Viewing the results

During the run, the real-time collection of data can be monitored in the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results are viewed in the Results Editor with data from each stage of the run located under its own tab.

- Click on the appropriate tab to view the results of the stage.

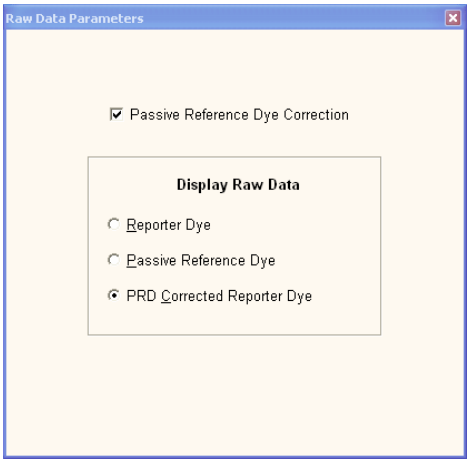
The baseline corrected graph will be displayed according to the parameters chosen.



4.5.3.1 Viewing and changing the parameters

- Click the **PAR** button next to the baseline correction graph to bring up the parameter settings for the analysis.

If any settings are changed, the data will be recalculated and the graphs and results table updated accordingly.



The settings or analysis method can also be changed by accessing the **Analysis Selection** box from the Results Editor main page.

If a PRD was assigned in the dye usage box, then the PRD correction options can be accessed by clicking the PAR icon next to the raw data graph.

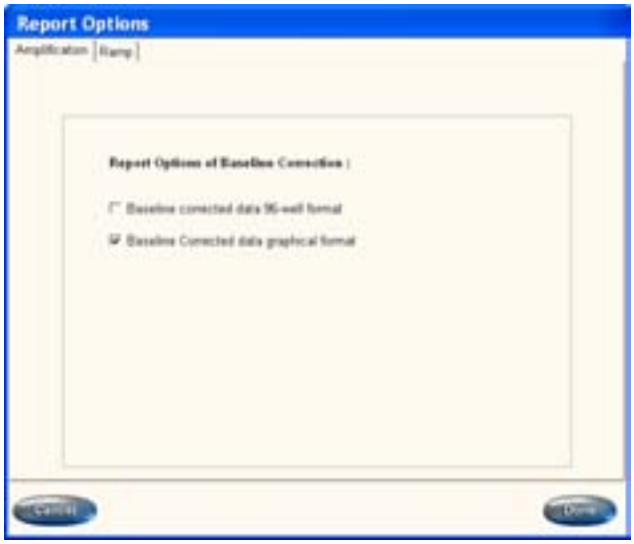
If the raw data graph is not shown, click the raw data graph icon.

4.5.4 PrimeQ Report

4.5.4.1 Display options

The report options can be changed from within the report tab of the Results Editor.

- Click on the report options icon, which will bring up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click **Done** to finish.



4.5.5 Quick guide to baseline correction analysis

1. In the Experiment or Results Editor Analysis Selection box, highlight the stage on which baseline correction is to be performed and click **Edit**.
2. In the Analysis Wizard Selection box, choose **Baseline** from the drop-down menu and assign a use next to the appropriate dye name. Click **Next**.
3. Choose whether to correct the results with a PRD, if a PRD was assigned in the dye usage box. Click **Next**.
4. Select a baseline correction option. Click **Next**.
5. Report options: select which results should appear in the report. Click **Next**.
6. Summary of analysis. Click **Finish** to return to the Experiment or Results Editor main screen.

4.6 Analysis method: Quantification

This analysis method has two general approaches: either to determine the 'absolute' concentration of an unknown sample by comparison of the quantification cycle (Cq) to a standard curve of known concentrations or else a relative value determined by the comparison of Cqs. The latter approach is useful in screening assays, for example, when an increase relative to a control sample provides sufficient experimental information such that an 'absolute' concentration is not required. Both approaches use Cq calculation as the basis of the quantification as reflected in the setup procedure detailed below.

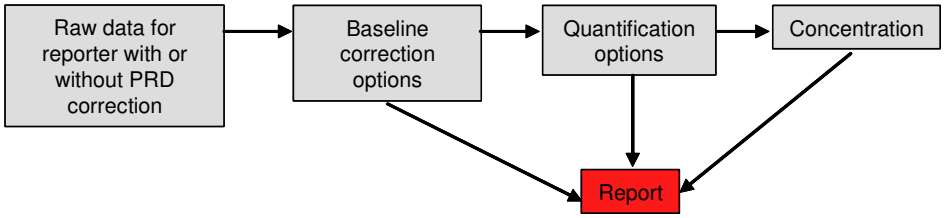
4.6.1 Assay requirements

- Need at least one reporter.
- Need at least two readings.
- Need dye defined as PRD for 'PRD correction' options to be available.
- Need at least two reporters for relative quantification methods.

This method allows baseline correction for amplification data followed by a quantification calculation using one of two methods. Results can then be given as a quantification cycle (Cq), an absolute concentration, if standards have been included, or relative concentration if more than one reporter is being used.

4.6.2 Setup

The diagram below gives an overview of Quantification analysis.



- In the Experiment Editor Analysis Selection box, highlight the stage name to which analysis is to be applied and click **Edit**. The Analysis Wizard will launch.
- Choose **Quantification** from the drop-down menu.
- Assign a use for the dye(s) employed in the assay. Click **Next**.
- The Quantification Wizard launches and leads the user through the quantification setup.

4.6.2.1 PRD

If a PRD was assigned in the dye usage box, the next screen displays an option for passive reference dye correction (see section 4.4). Clicking **Next** leads through to the Baseline Correction screen.

4.6.2.2 Baseline correction

See section 4.5. Choose the method as required and set the values as appropriate.

4.6.2.3 Cq calculation

The crossing line is a best-fit of where the reaction efficiency is at its highest and most constant for each reaction curve. The quantification cycle (Cq) is the point at which an amplification curve intercepts the crossing line and measuring fluorescence at the Cq for each well will allow the most reliable well-to-well comparison (and when compared to standards, the quantification of unknowns). The Cq calculation screen provides two options for Cq calculation:

- **Fit points: (Default option):** Involves setting a noise and crossing threshold. Useful for quantifying low copy number targets.
- **First derivative maximum:** Calculates Cq values automatically based on the point at which the rate of change is fastest. As this method uses the shape of the curve to determine Cq, there is no user-input in the analysis of the data. This method can be useful for analysing high copy number templates (over 1000 copies).

4.6.2.4 Fit points

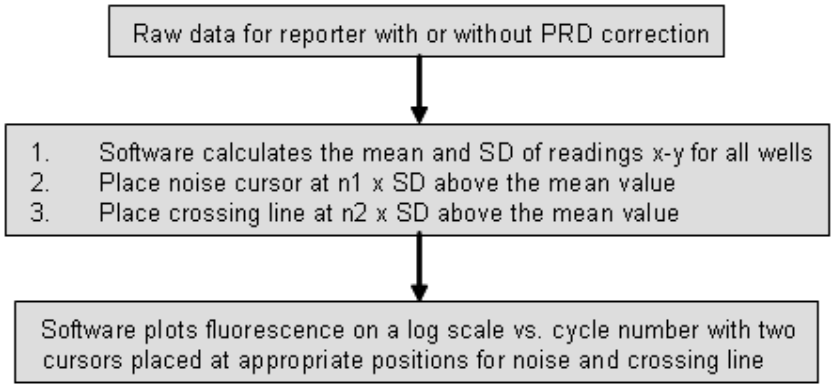
This approach sets a crossing line at a specified distance above a noise threshold. Where the amplification curve intercepts the line, a specified number of data points above the line are used in a linear regression calculation to determine the Cq.

Procedure:

- Select fit points method
- Set noise threshold
- Set crossing line
- Set number of fit points
- Select whether to display fit points on the graph

Setting the noise threshold and crossing line

Overview of procedure:



The noise threshold and crossing line are placed on the amplification graph either at positions specified by the user or using the default settings.

Default values:

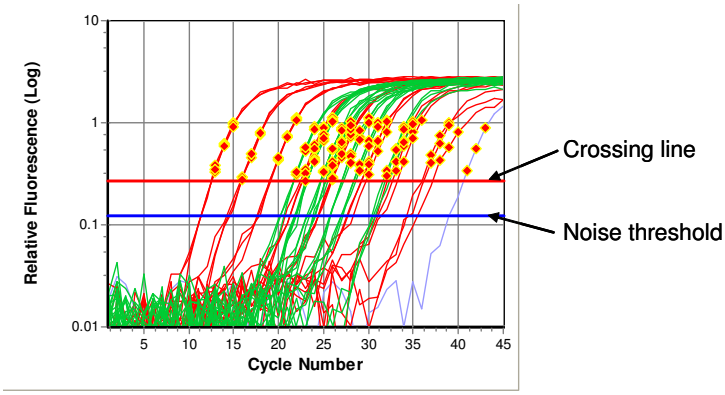
Noise threshold cursor: n1 = 4 SD above average of all readings x to y

Crossing line cursor: n2 = 10 SD above average of all readings x to y

x = 3 (or 1 if number of readings less than 10)

y = 10 (or 2 if number of readings less than 10)

Once data has been obtained and displayed in the Results Editor, the thresholds will appear as two horizontal cursors on the plot (blue for the noise threshold and red for the crossing line). Fits points are shown as orange points above the crossing line. If necessary, the user may manually slide the cursors to positions better suiting the data.

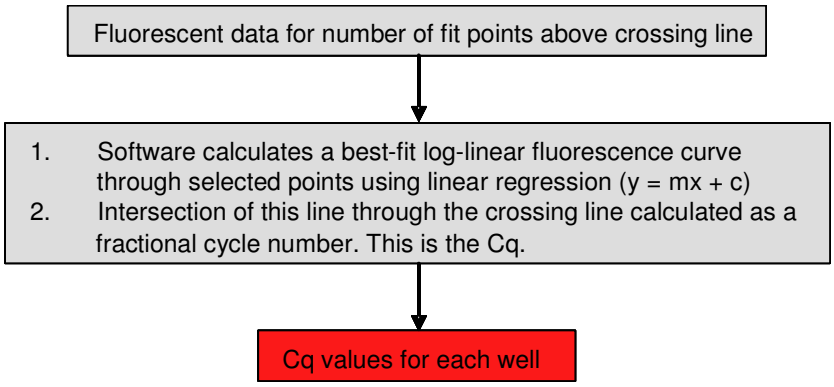


- The user only has to choose where to place the cursors (which are placed automatically and then movable by the operator) – all calculations are performed automatically by the software.
- The results (with or without correction) are plotted on a scale of log fluorescence vs. cycle number. Although some fluorescence values may be negative, this has no effect on the accuracy of the data.
- Up to five orders of magnitude of fluorescent data below the maximum value will be plotted with the zero and negative values discarded.

Calculating the quantification cycle

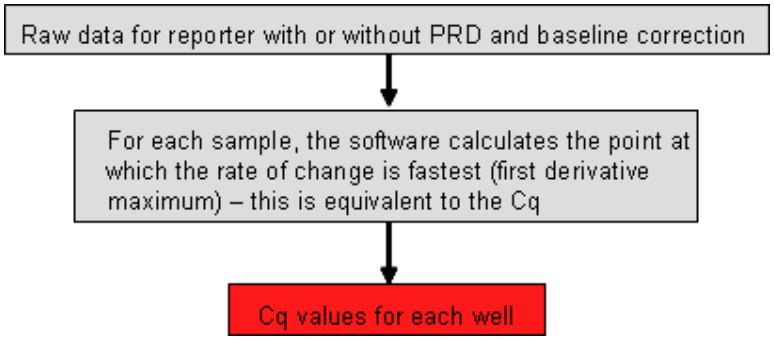
Fit points are a defined number of fluorescent points found on the curve just above the crossing line. The points are used in a linear regression calculation and the interception of the linear regression through the fit points and crossing cursor provides a value for the quantification cycle. This will be a fractional number calculated for each well. All the user has to do is set the number of fit points to be included in the calculation and the software does the rest.

Overview of procedure:



4.6.2.5 First derivative maximum

Overview of procedure:



Procedure

- In the Cq calculation window of the Quantification Wizard, click **First Derivative Maximum** and then **Next**.

As this method calculates Cq by defining the point of fastest rate of change in fluorescence, no noise threshold or crossing cursor has to be set. Therefore, the wizard links straight through to the Report Options.

4.6.2.6 Report Options and Summary

- Clicking **Next** leads through to the **Report Options** screen. Click the appropriate boxes to select which data is to be displayed in the report.
- Click **Next** to view a summary of the setup.
- Click **Back** to change any settings or **Cancel** to abort the procedure.
- Click **Finish** to complete the setup.

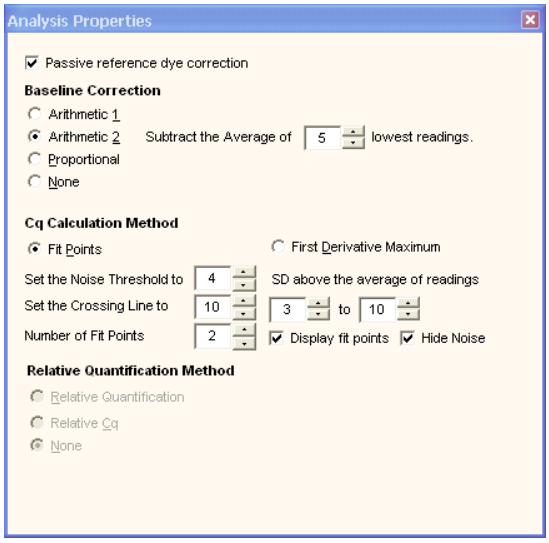
4.6.3 Viewing the results

During the run, the real-time collection of data can be monitored in the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.

4.6.3.1 Viewing and changing the analysis parameters

Clicking the **PAR** button on any of the graphs displays the settings for the analysis method condensed into a single pane. The user can change any of the parameters, and in doing so, the changes will be reflected immediately in the adjacent graphs.



Changes to the analysis parameters can also be made via the **Analysis Selection** box on the main page of the Results Editor.

If a PRD was assigned in the dye usage box, then the PRD correction options can be accessed by clicking the **PAR** icon next to the raw data graph.

- Click the raw data graph icon if the raw data graph is not displayed.

4.6.4 PrimeQ Report

The report options can be changed from within the report tab of the Results Editor. Click on the report options icon, which will bring up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click **Done** to finish.

4.6.5 Using Cq values

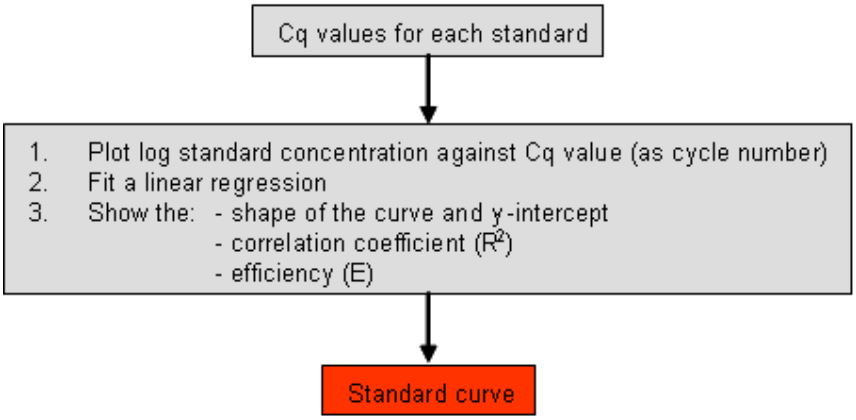
Cq values can be used in a number of different ways:

- Plot the Cqs of standards against log concentration to produce a standard curve – concentration of unknowns can be read off using their Cq.
- Compare Cq values from well-to-well.
- Compare the Cqs from two different reporters in the same well.
- Compare the concentration of the two different reporters in the same well.

4.6.6 Comparing Cq values using a standard curve

If there are defined standards in the plate layout, the Cq for each can be plotted against the log of its concentration to produce a standard curve. The concentration of an unknown can then be extrapolated from the graph on the basis of its Cq.

Overview of procedure:



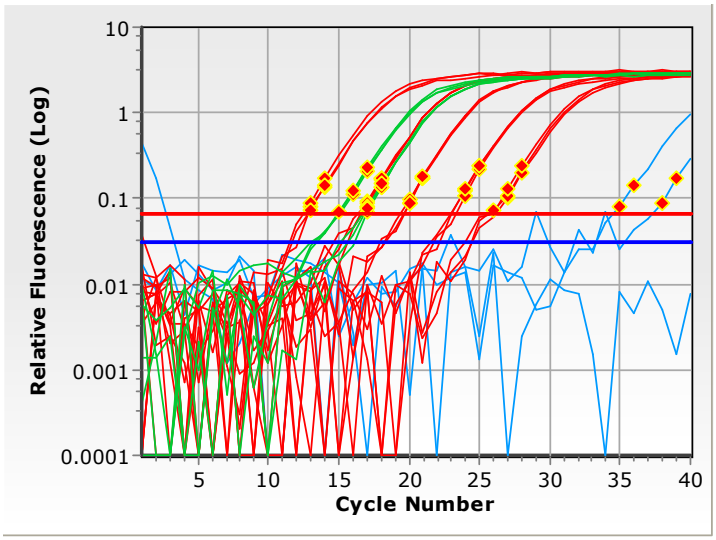
The experiment is set up using the Quantification Wizard as detailed above. The experiment must include a dilution series of standards in the plate layout by which to compare the unknowns. The concentration of each standard should be added to the **Well Information** table in the Plate Layout Editor (can be accessed through the Results Editor main screen, post-run).

- If less than two standards have been assigned in the plate layout, a message in the Results Editor will alert the user to the fact that there are insufficient standards.
- If no standards have been assigned, no standard curve graph will be displayed.

4.6.6.1 Quantification cycle graph (fit points analysis only)

- Click the Cq icon if the quantification cycle graph is not displayed.

The results (with or without correction) are plotted on a scale of log fluorescence vs. cycle number. Although some fluorescence values may be negative, this has no effect on the accuracy of the data.



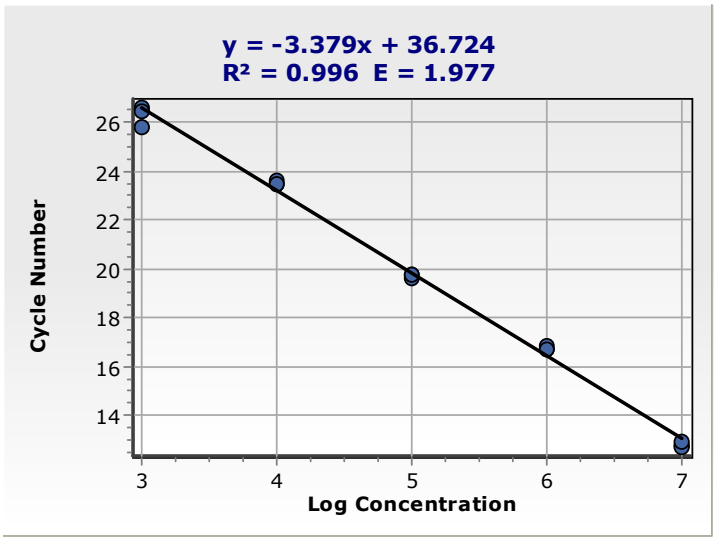
The blue line represents the noise threshold and the red line the crossing line.

The user can manually adjust the position of the lines by dragging with the mouse to positions better suiting the data.

The position can also be changed from the PAR box accessed by clicking on the **PAR** button next to the Cq graph.

Changing the position of the cursors will update the graphs. This can be useful for adjusting the best fit such that the R^2 value (correlation coefficient) is as close to 1 as possible i.e. a perfect correlation (see The standard curve below).

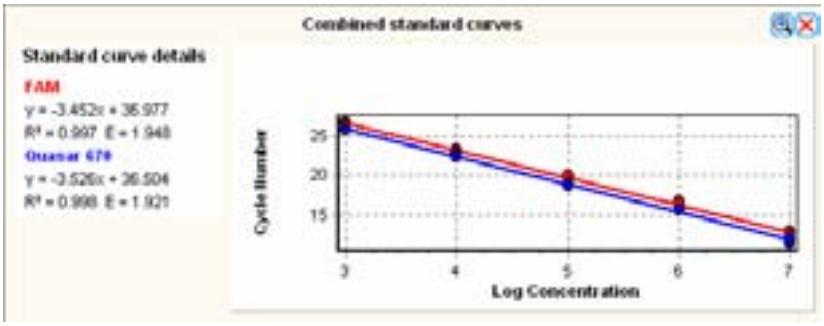
4.6.6.2 The standard curve



Linear regression is used to generate a straight line, y = mx + c whereby:

- y = the slope, ideally this should be approximately -3.32.
- c = the value at which the line intercepts the y axis.
- R^2 = Correlation coefficient (mean squared error of the determination) – a perfect correlation has a value of 1.
- E = efficiency of the reaction whereby E = $10^{-1/slope}$. The ideal value is 2 for 100% efficiency (i.e. a doubling of target every cycle). A value less than 2 suggests that the PCR is sub-optimal while above 2 suggests the amplification of non-specific products. When comparing two reporters in one well, it is important for both PCRs to have a near equal efficiency (typically in the range of 1.95 and 2.05).

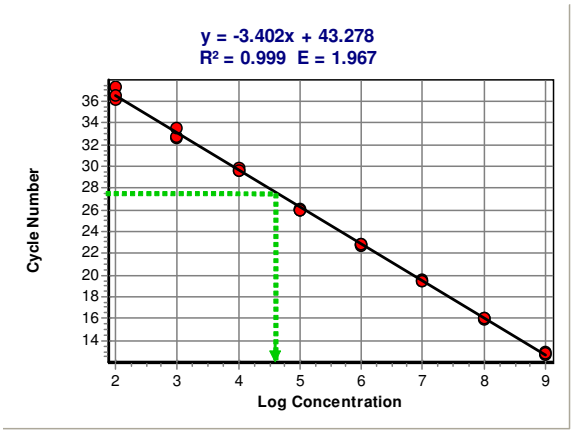
If there is more than one reporter dye with standards (for example in Relative Quantification) then the standard curve for each reporter can be displayed on the same graph for comparison.



This is useful for optimization of reactions in preparation for multiplexing. See section 4.11.

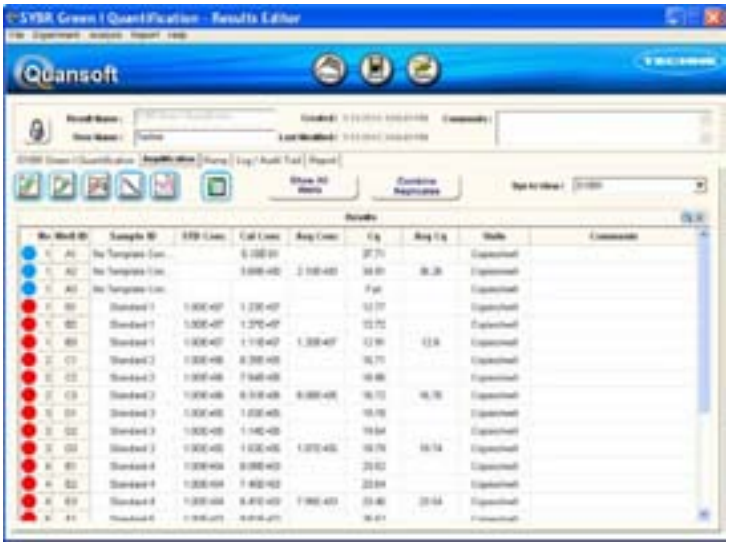
4.6.6.3 Calculating unknowns

The standard curve can be used to calculate the starting concentration of unknowns by reading their Cq values from the graph. In Quansoft this is performed automatically and the calculations are displayed in the results table.



4.6.6.4 Results table

Concentrations are calculated for the unknowns based on a comparison of Cqs with standards of known concentration. The results are displayed in the results table.



Column headings:

- **No:** Well number.
- **Well ID:** Location of well.
- **Sample ID:** User-supplied or default name of well.
- **STD Conc:** Concentration of standard (not shown if < 2 standards in plate layout).
- **Cal Conc:** Calculated concentration of sample.
- **Avg Conc:** Mean concentration obtained for all replicates of a sample
- **Cq:** Quantification cycle for sample.
- **Avg Cq:** Mean Cq obtained for all replicates of a sample.
- **Units:** Unit of concentration.
- **Comments:** User inputted text.

4.6.7 Comparing Cqs in relative quantification

Cqs are also used for relative quantification i.e. the comparison of two different reporters in the same well. Although this approach can be performed either with or without a standard curve, it is particularly useful for screening assays where it is necessary to compare a fold difference of sample B to a calibrator sample A, for example. In such an assay, information about absolute amounts is not required as the value relative to the calibrator provides the necessary information.

Setup procedure

- In the Analysis Wizard, select **Quantification** as the analysis method and assign reporters (note: two reporters must be assigned for relative quantification). Click **Next**.
- Set a baseline correction method.
- Set a Cq calculation method as described above.

The user will then be asked to choose a relative quantification method.

- **Relative quantification:** Compares the concentration of two reporters – requires standards.
- **Relative Cq:** Compares the Cqs of two reporters – requires no standards.

4.6.7.1 Relative quantification

Selecting Relative Quantification brings up the following screen:

- Select which reporters are to be compared using the drop-down menu. The concentration of the **first** reporter selected will be divided by that of the **second**.

Clicking **Next** takes the user through to Report Options window as before.

- Click through to the Summary window and click **Finish**.

4.6.7.2 Viewing relative quantification results

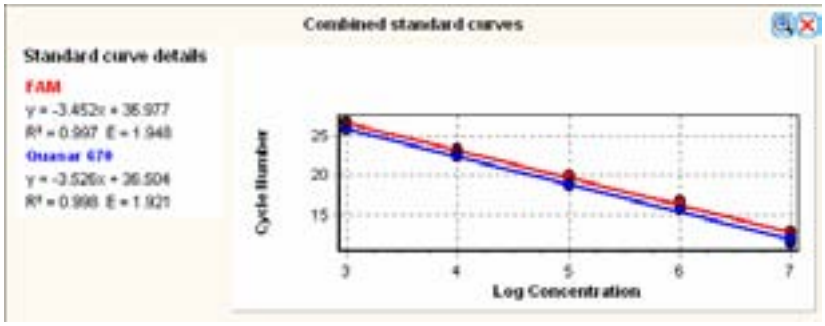
During the run, the real-time collection of data can be monitored in the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.

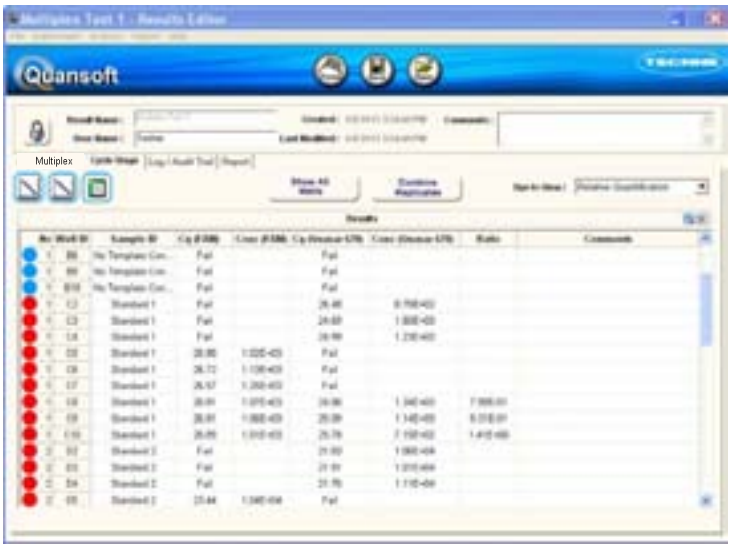
The Results Editor will display relative quantification data consisting of the plate layout and the standard curve for each reporter.

- Scroll down the **Dye to View** options to display the full results for each individual reporter. This will appear in the same format as displayed when using a single reporter in that there is a baseline correction graph (if chosen in the analysis setup), a quantification cycles graph and the standard curve.

- Scroll down the Dye to View and select **Combined standard curves** to display the standard curves for each reporter on the same plot. The details of each standard curve can be useful when optimizing reactions in preparation for multiplexing.



The results table displays the calculated results.



Column headings:

- **No:** Well number.
- **Well ID:** Location of well.
- **Sample ID:** User-supplied or default name of well.
- **Cq (Dye 1):** Quantification cycle for Dye 1.
- **Conc (Dye 1):** Calculated concentration of sample for Dye 1.
- **Cq (Dye 2):** Quantification cycle for Dye 2.
- **Conc (Dye 2):** Calculated concentration of sample for Dye 2.
- **Ratio:** Dye 1 divided by Dye 2.
- **Comments:** User inputted text.

4.6.7.3 Relative quantification cycles

The relative Cq method allows the user to perform relative quantification without using a standard curve. Instead, the Cq of an unknown sample is normalized to a known sample e.g. a reference gene (REF) amplified at the same time from the same sample. For many gene expression studies it is not necessary to determine the absolute amount of a target as evidence of a relative increase or decrease in expression compared to a reference sample (calibrator) proves sufficient. The expression level of the gene of interest in the calibrator sample is defined as 1x, with levels in the unknowns reported relative to it.

Choosing **Relative Cq** in the Relative Quantification window brings up the settings box.

The user can select which reporters to compare using the drop-down box shown above. The first is usually the gene of interest and the second the REF gene amplified from the same sample. The Cq of the second reporter selected will be subtracted from that of the first and the resulting ΔCq value can then be compared to that of a calibrator (if a reference sample has been defined as a calibrator, **CAL**, in the plate layout). The calculation, which is performed by the software, can be summarized as follows:

- Calculate Cq for each sample (using either fit points or first derivative maximum).
- Calculate the ΔCq for each sample e.g. ΔCq = Cq reporter – Cq REF.
- If a calibrator sample is present, then assign this sample as a **CAL** sample type in the plate layout.
- Calculate ΔΔCq: ΔΔCq = ΔCq sample – ΔCq calibrator.
- Calculate $2^{-\Delta\Delta Cq}$

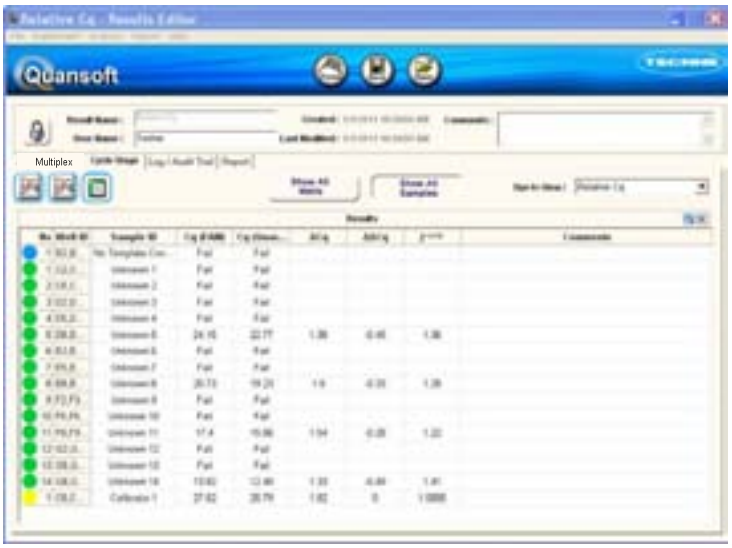
This value represents the amount of target normalized to a control gene (REF) and relative to a calibrator (CAL) sample. If this number is more than 1, it represents a fold increase in expression over the calibrator which in theory has no upper limit. However, the user must be aware that a decrease can only be represented by a value between 0 and 1 such that a seemingly small decrease may in fact represent a significant decrease in expression relative to the calibrator.

The same principle applies for the report options as for relative quantification. The results table shows the Cq calculations.

4.6.7.4 Viewing comparative Cq results

The Results Editor will display the comparative Cq data consisting of the plate layout and the amplification curves for both reporters.

- Dye to view options offer relative quantification cycles or individual reporter results and graphs.
- Changing the Dye to View from Relative Cq to a single reporter displays the baseline corrected graph (if chosen in the setup) in addition to the quantification cycles graph for the individual dyes.



Column headings:

- **No:** Well number.
- **Well ID:** Location of well.
- **Sample ID:** User-supplied or default name of well.
- **Cq (Dye 1):** Quantification cycle for Dye 1.
- **Cq (Dye 2):** Quantification cycle for Dye 2.
- **ΔCq:** Calculated ΔCq.
- **ΔΔCq:** Calculated ΔΔCq.
- **$2^{-\Delta\Delta Cq}$:** Calculated $2^{-\Delta\Delta Cq}$
- **Comments:** User inputted text.

4.6.8 Quick guide to quantification analysis

4.6.8.1 Quantification

1. In the Experiment or Results Editor Analysis Selection box, highlight the stage on which quantification analysis is to be performed and click **Edit**.
2. In the Analysis Wizard Selection box, choose **Quantification** from the drop-down menu and assign a use next to the appropriate dye name. Click **Next**.
3. Choose whether to correct the results with a PRD if a PRD was assigned in the dye usage box. Click **Next**.
4. Select a baseline correction option. Click **Next**.
5. Choose a Cq calculation method. If choosing the fit points method, set the noise threshold, crossing line and number of fit points. Click **Next**.
6. Report options: select which results should appear in the report. Click **Next**.
7. Summary of analysis. Click **Finish** to return to the Experiment or Results Editor main screen.

Relative quantification

1. Follow steps 1–3 as above but assign two reporters from the dye usage menu. Click **Next**.
2. Select a baseline correction option. Click **Next**.
3. Choose the Cq calculation method. Click **Next**.
4. Select the relative quantification method required. Choose from Relative Quantification or Relative Cq and select which reporter is to be compared to which. Click **Next**.
5. Report options: select which results should appear in the PrimeQ report. Click **Next**.
6. Summary of analysis. Click **Finish** to return to the Experiment or Results Editor main screen.

4.7 Analysis method: Dissociation curve

Dissociation curve analysis can add to the information obtained from the PCR. Also known as melting curve analysis, it measures the temperature at which the DNA strands dissociate (i.e. the melting temperature or Tm). Using an intercalating dye such as SYBR® Green I and increasing the temperature in small increments, the PCR products can be seen to 'unzip' at a specific temperature. Since the melting temperature is characteristic of the GC content, length and sequence of a DNA product, it is a useful tool in product identification.

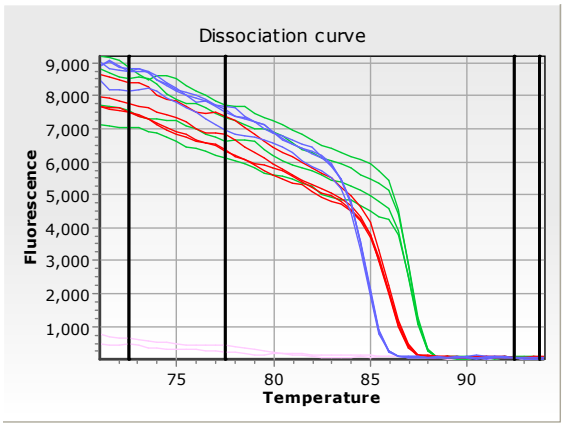
Dissociation curve analysis has applications in:

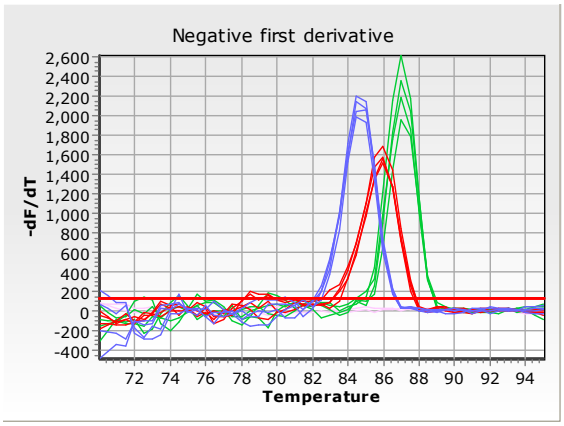
- Identifying and characterizing PCR products.
- Genotyping and mutation analysis using hybridization probes.
- Optimizing PCR: If the Tms of a PCR product and primers are known, the annealing temperature can be kept above the Tm of the primer-dimers but below the Tm of the specific PCR product.
- Estimating relative concentration of products: Peak area analysis can provide an indication of the relative amount of each product.

Dissociation curve analysis is an inversion of the PCR amplification method in as much as a cycle-to-cycle decrease in fluorescence is seen as opposed to an increase.

- At the start of an analysis, the reaction is at a relatively low temperature and the fluorescence signal high.
- As the temperature slowly ramps upwards, the fluorescence will drop more rapidly as the reaction reaches the melting point.
- Pure, homogenous PCR products will produce a single sharply defined melting curve with a sharp peak, while primer-dimers will generally melt at a lower temperature with a broader peak.

Dissociation peak analysis is a plot of the negative first derivative of the dissociation curve which shows a characteristic peak for each product (the derivative is the negative of the rate of change in fluorescence as a fraction of temperature). By taking the derivative of the dissociation curve as opposed to using the raw data, identifying the Tm is made easier as a peak is produced:



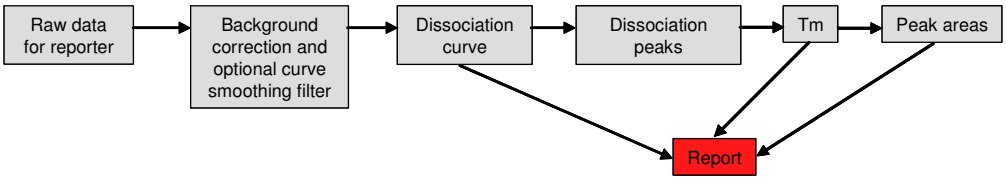


The Tm is the maximum point of the peak and the temperature at which 50% of the product has melted. Peak area analysis measures the area under a peak and can provide an indication as to the relative amounts of each product. Each analysis follows on from the next so to calculate peak areas, for instance, it is necessary to have already analysed the dissociation peaks and Tms.

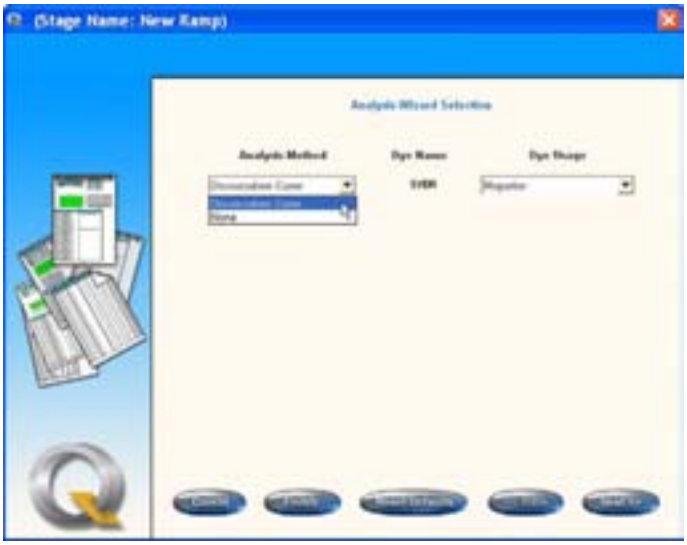
4.7.1 Assay requirements

- Need at least one reporter dye.
- Need a stage in the program with a ramp read (with at least two readings).

4.7.2 Setup



- Program a ramp read into the PCR program as described in section 3.4.3.6.
- In the Analysis Selection box in the Experiment Editor, click on the name of the ramp stage and then press **Edit**.
- This will lead through to the Analysis Wizard Selection box. Select **Dissociation Curve** in the drop-down menu and select dye usage.



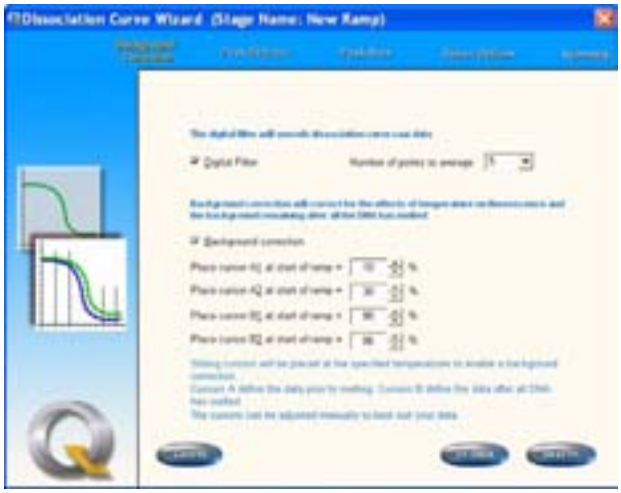
- Click **Next** and the Dissociation Curve Wizard will launch.

The wizard will lead the user through the setup:

- **Background correction:** Corrects for effects of temperature on fluorescence and the background before and after all the DNA has melted.
- **Digital filter:** Smoothes the dissociation curve raw data (but does not affect the calculated results).
- **Peak detector:** Finds specified number of peaks and records the dissociation temperature (Tm).
- **Peak area:** Calculates the area under the peaks detected.

4.7.2.1 Background correction

The first window of the wizard allows setting of background correction parameters.

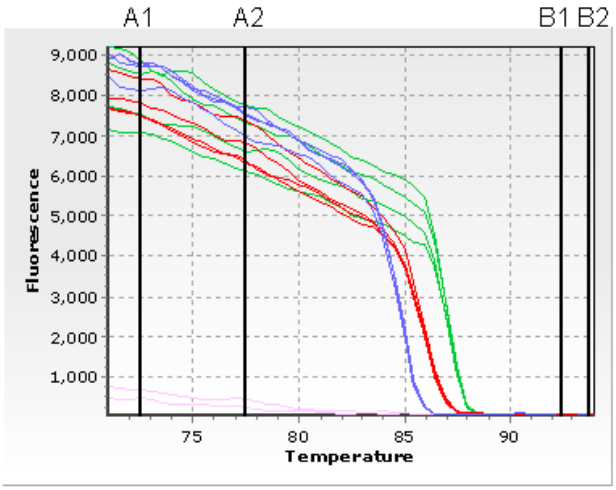


Digital filter

This is an option to smooth the raw data before it is presented. It is carried out using a Savitsky-Golay curve smoothing algorithm, which fits a third order polynomial to a number of points either side of the data to be smoothed. This is similar to averaging points either side of a centre point but because a third order polynomial curve is used, the profile of the curve is retained. The user can set the number of points on which to smooth the data in the drop-down box. **The smoothing is for display purposes only** – subsequent calculations use only the raw data.

Background correction

Select this option to correct the data for the effects of temperature on fluorescence and for the background remaining after all the DNA has dissociated. The default is **ON** since this has to be enabled for peak curve analysis. Background correction is applied to a plot of fluorescence vs. temperature using two sets of user-defined cursors. Settings boxes are shown in the Dissociation Curve Wizard window.



- Click the background correction icon if the graph is not displayed:

A1–A2: These cursors determine the effect of temperature on the fluorescence signal. They should be positioned in the flat fluorescence region measured before any product has melted. The default values are:

- A1: Start temperature +10% ramp
- A2: Start temperature +30% ramp

For example, if the temperature ramps for 30°C between 50°C and 80°C, 10% would equate to 3°C and A1 would be positioned at 50 + 3 (53°C); 30% equates to 9°C and so A2 would be positioned at 50 + 9 (59°C) and so on. These cursors should be positioned as far apart as possible without including any of the dissociation data.

B1–B2: These cursors determine the remaining fluorescence background after the DNA has dissociated. They are placed in the flat fluorescence region that occurs after the melt. The default values are:

- B1: Start temperature +90% ramp
- B2: Start temperature +95% ramp

The cursors are applied to the fluorescence vs. temperature plot once the data has been collected. Within the Results Editor, the user can adjust the cursors manually to best suit the data. The background rate of change over the user-defined region A is calculated automatically for each well using linear regression. This generates a correction index for each well which is used to correct the data until it falls below a pre-defined point. The correction for region B is calculated taking the average of all readings in region B and subtracting this value from the data for each well.

4.7.2.2 Peak detection

Clicking **Next** from the background correction screen leads to the peak detection options.

Principle

The plot of dissociation peaks involves a calculation that takes the negative of the rate of change in fluorescence as a function of temperature (-dF/dT). This approach uses a second order polynomial equation to calculate a best-fit curve through the data point plus one either side. The rate of change for each value can then be calculated by differentiating the equation.

The highest point of each peak (Tm) can then be calculated using the second order polynomial equation of the three points around the peak in a similar manner to the calculation for first derivative maximum for Cq calculation in quantification analysis. The best-fit equation is differentiated and solved for dy/dx = 0 to obtain temperature (Tm) at the peak.

Methods

The Dissociation Wizard provides two options for peak detection:

- The software is instructed to automatically find a number of peaks mathematically (**Auto peak detect**).
- The user manually places cursors onto the graph of peak data (**Manual peak detect**).

4.7.2.3 Auto peak detect

The user defines the settings that the software will use to score for peaks.

- **Number of peaks to find:** Up to a maximum of four.
- **Threshold filter:** The user sets a threshold value to limit the size of the peaks detected (see below). The default is 5%.
- **Order by peak height or Tm:** A list of peaks and height are produced for each well and then filtered to remove any peaks less than the noise level. The results can be ordered according to either the peak height (largest first) or temperature (Tm). If there are replicates, each well will be treated individually.

4.7.2.4 Manual peak detect

To use manual peak detect, select this option on the Dissociation Wizard Peak Detector screen.

- **Number of peaks to find:** Up to a maximum of four.
- **Bin threshold:** The user can specify a temperature range (°C) in which a peak will be considered valid.
- **Threshold filter:** The user sets a threshold value to limit the size of the peaks detected (see below). The default is 5%.

Once the data has been collected, the specific number of cursors will be present on the dissociation peak graph in the Results Editor.

To select further peaks:

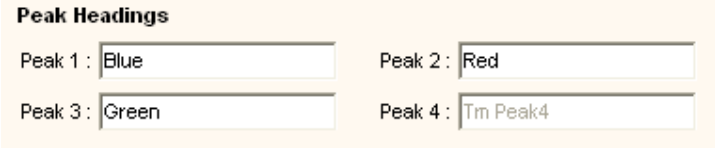
- Click on the **PAR** button next to the dissociation peak graph.
- Enter the number of peaks required in the **Peak Detector** window of the Dissociation curve parameters.
- Click on each cursor using the mouse and drag each detection cursor to the desired position on the graph.

Peak Headings

Each peak cursor can be individually named by typing a name in the appropriate Peak Headings box.

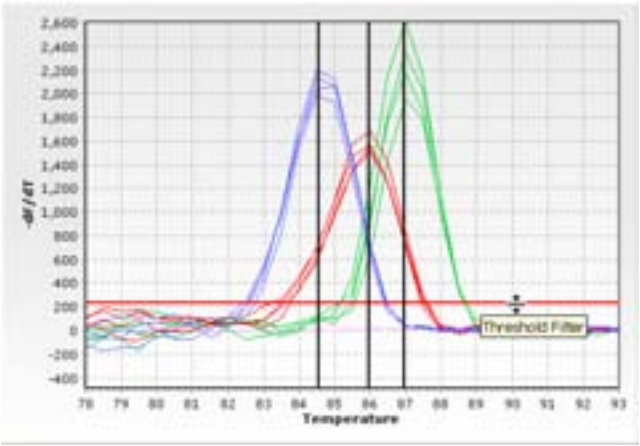
- Click on the **PAR** button to view the **Peak Headings** box.
- Type in a name for the peak.

The new name will then appear in the Results Table column heading and in the Report. This may facilitate identification of peaks corresponding to different PCR products.



Threshold filter

The Threshold filter appears as a red horizontal sliding cursor across the dissociation peaks graph at the default setting of 5%. This parameter limits the size of the peaks detected – peaks smaller than the specified threshold % relative to the tallest peak are not recorded. For example, if a well's largest peak has a height of 1000 on the dissociation peak graph (-dF/dT vs. temp) and the threshold is set to 5%, any peaks with a height of less than 50 are assumed to be noise and ignored.



The cursor can be moved manually to increase or decrease the threshold value for peaks detected. The new cursor position will be updated in the **PAR** box.

In a small number of cases, a Tm for a peak may appear in the Results Table even when the cursor is placed just above peak. This is due to the mathematical curve fitting algorithm used to calculate the Tm. The peak can be excluded by raising the cursor slightly higher.

Bin threshold

The user can specify a temperature range (ºC) either side of a cursor in which a peak will be considered valid. For example, if the bin threshold is set to 80℃ with a range of 0.5℃, then all peaks with a Tm between 79.5 and 80.5℃ will be considered in the results. The default is 0.5℃.

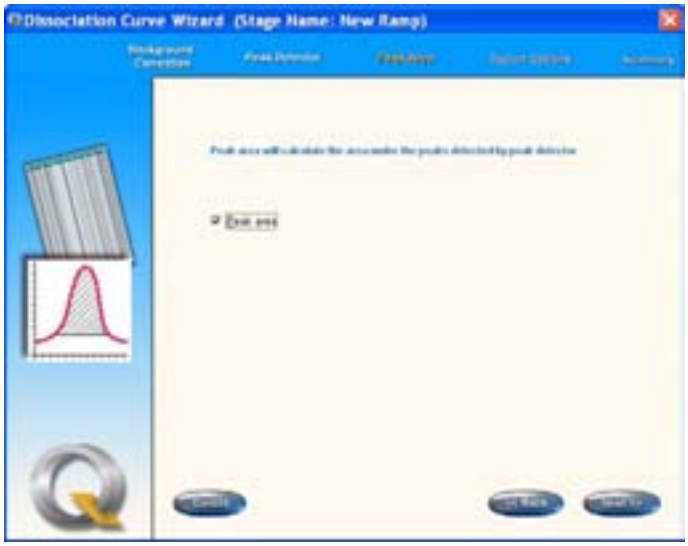
Peaks within the valid +/- temperature range are sent to the peak column results table. If a peak is present but falls outside of the valid range, then the box is left blank such that there will be as many peak columns as there are cursors defined. Replicates are not averaged.

No	Well ID	Sample ID	Blue (°C)	Red (°C)	Green (°C)
1	A1	No Template Contro...			
1	A2	No Template Contro...			
1	C5	Red 1		85.94	
1	C6	Red 1		85.95	
2	D5	Red 2		85.90	
2	D6	Red 2		85.86	
1	A3	Green 1			87.12
1	A4	Green 1			87.02
2	B3	Green 2			87.05
2	B4	Green 2			86.99
1	E7	Blue 1	84.68		
1	E8	Blue 1	84.68		
2	F7	Blue 2	84.68		
2	F8	Blue 2	84.75		

- If the cursors are very close together then it is possible that their validity ranges may overlap. In this case, the space between should be shared equally so overlap no longer occurs e.g. if there is a cursor of 80℃ and one at 81℃ and the validity range is 1.5℃, then cursor 1 should have a validity range of 79.0 to 80.5℃ and cursor 2, a range of 80.5 to 82.0℃.
- When the experiment is first run, there is no data shown in the results table as peaks have yet to be defined. Once the positions of the cursor and their names have been set, these can be saved as part of the Experiment file to allow a repeat run with the same parameters.

4.7.2.5 Peak area

When peak detection parameters have been set in the Dissociation Curve Wizard, clicking **Next** leads through to the Peak Area screen.



The peak area option calculates the area under the selected peak(s) of interest and can provide an indication of the relative amounts of each product in a sample.

The peak area of an unknown sample can be compared against the peak area of a known standard amplified in the same tube. A Gaussian fit is performed for each peak of interest and the area calculated is presented in a table similar to that for Tm results.

No	Well ID	Sample ID	Tm Peak1 (°C)	Area Peak1	Tm Peak2 (°C)	Area Peak2	Tm Peak3 (°C)	Area Peak3
1	A1	No Template Contro...						
1	A2	No Template Contro...						
1	C5	Red 1			85.94	2,906		
1	C6	Red 1			85.95	2,894		
2	D5	Red 2			85.90	3,571		
2	D6	Red 2			85.86	3,168		
1	A3	Green 1					87.12	3,425
1	A4	Green 1					87.02	3,800
2	B3	Green 2					87.05	4,113
2	B4	Green 2					86.99	4,603
1	E7	Blue 1	84.68	3,939				
1	E8	Blue 1	84.68	4,360				
2	F7	Blue 2	84.68	4,141				
2	F8	Blue 2	84.75	4,045				

- Choose the **Peak Area** option by checking the box.

4.7.2.6 Report Options and Summary

- Clicking **Next** leads through to the Report Options screen, which allows the user to decide how the data appears in the PrimeQ report.
- Click **Next** to view a **Summary** of the setup.

- Click **Back** to change any settings or **Cancel** to abort the procedure.
- Click **Finish** to complete the set up.

4.7.3 Viewing the results

During the run, the real-time collection of data can be monitored on the Run screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab. Results of the ramp will be displayed under the tab in which a ramp read was defined.

4.7.3.1 Viewing and changing the parameters

Click the **PAR** button next to the graphs to bring up the settings box for dissociation curve analysis. If any settings are changed, the data will be recalculated and the graphs and results table updated immediately.

The settings or analysis method can also be changed by accessing the **Analysis Selection** box from the Results Editor main page.

4.7.4 PrimeQ Report

The report options can be changed from within the report tab of the Results Editor. Click on the **Report Options** icon, which will bring up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click **Done** to finish.

4.7.5 Quick guide to dissociation curve analysis

- In the Experiment or Results Editor Analysis Selection box, highlight the stage on which Dissociation Curve is to be performed and click **Edit**.
- In the Analysis Wizard Selection box, choose **Dissociation curve** from the drop-down menu and assign a use next to the appropriate dye name. Click **Next**.

- Background correction screen: Choose whether to smooth the data with a digital filter and define where to position the cursors for correction. Click **Next**.
- Peak Detection: Choose to detect peaks automatically or manually. Set the number of peaks and threshold filter for automatic detection or the bin threshold and threshold filter for manual detection. Click **Next**.
- Peak area: Check the box to have peak area calculated from the data. Click **Next**.
- Report options: Decide which data to display in the PrimeQ report. Click **Next**.
- Summary of analysis. Click **Finish** to return to the Experiment or Results Editor main page.
- The updated method appears in the Analysis Selection box in the Experiment or Results Editor main screen.

4.8 Analysis method: Plus-minus scoring

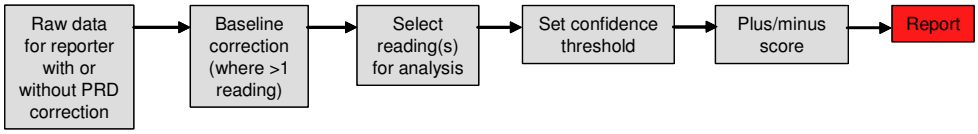
This type of assay is used to record the presence or absence of a PCR product. Input data can either be kinetic (whereby the number of readings is >1) or end-point (a reading taken at the end of the run). The assay uses fluorophore chemistry in the same way as in the other assays such that the fluorescence produced during the reaction will be proportional to the number of target DNA molecules.

Plus-minus assays can be performed with or without the use of an internal positive control (IPC), although its use can prove helpful to ensure that a failed PCR is not mistaken for a negative result. A no template control (NTC) is used to set confidence thresholds above which all unknowns will be scored as positive and below which they will be scored as negative. Samples between the two threshold values are scored as undetermined. With no user input, the software will automatically analyse the raw data (or baseline corrected data) to measure the difference in fluorescence between the unknown samples and NTC.

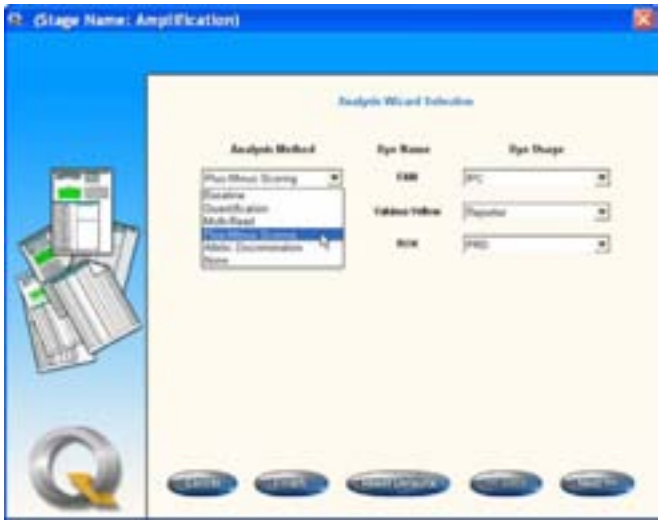
4.8.1 Assay requirements

- Need at least one reporter dye.
- Need at least one reading.
- Need a dye defined as a PRD for PRD correction options to be available.
- Need at least two readings for baseline correction to be available.
- Need a no template control (NTC).

4.8.2 Setup



- In the Analysis Selection box, highlight the stage name for analysis to be applied and click **Edit**. The Analysis Wizard will launch.
- Select **Plus-Minus Scoring** from the drop-down menu in the Analysis Method selection box. Assign dye usage and click **Next**. The Plus-Minus Wizard will launch.



- If a PRD was assigned in the dye usage menu, the next screen will offer the option of PRD correction. Click **Next**.

4.8.2.1 Baseline correction

This option is only available if the data is kinetic (i.e. > 1 reading). The choice of baseline correction method is similar to that shown in the Baseline Correction Wizard (section 4.5) with the exception that Proportional is excluded. Click **Next** when the baseline correction is set.

4.8.2.2 Plus-minus analysis method

This screen allows the user to choose which readings are to be used for results scoring.

- **End-point (default):** Uses the last reading only (we recommend that >1 reading is used for accuracy).
- **All readings:** Averages all readings in the stage
- **Last readings**: Averages a user-specified number of last readings (the default is 5, or 1 if there are less than 5 readings)
- **Specify range:** Specify a range of readings to be averaged that best suit the data (the default is the last reading).

4.8.2.3 Threshold settings

Clicking **Next** leads through to the **Threshold Settings** screen. In plus-minus scoring, a no template control (NTC) is used to set confidence thresholds above which all unknowns are scored as positive and below which as negative. Samples between the two threshold values are scored as undetermined. This screen provides options for these threshold settings.

- Set the threshold range using the scroll-down menu.

The default settings are:

Upper threshold: Average of NTC samples + n SD (default 6 SD)

Lower threshold: Average of NTC samples + n SD (default 3 SD)

4.8.2.4 Report Options and Summary

- Click **Next** to lead through to the **Report Options** screen. The default is for the plus/minus table to be displayed but an option for a scaled data graph displaying the confidence thresholds is also available.
- Click **Next** to view a **Summary** of the setup.

- Click **Back** to change any settings or **Cancel** to abort the procedure.
- Click **Finish** to complete the set up.

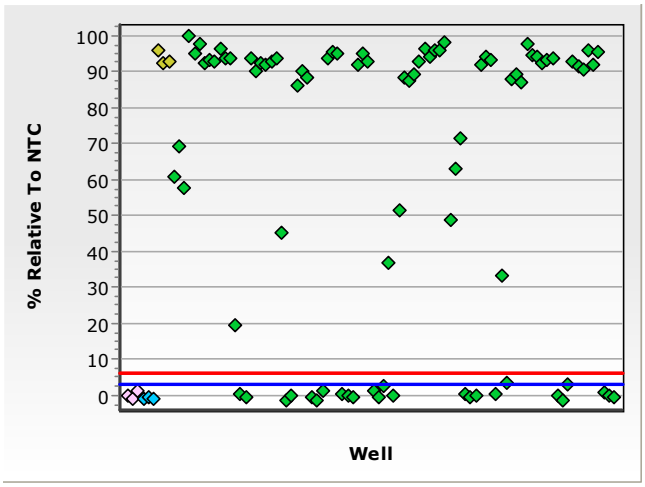
4.8.3 Viewing the results

During the run, the real-time collection of data can be monitored on the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.

Clicking an individual well or a selection of wells in the plate layout will highlight just the selected well(s) on the plus-minus graph. Clicking the Show all wells button will re-select all wells.

The plus-minus graph



If the plus-minus graph is not displayed, click on the icon to bring up the data:

The plus-minus graph displays the values as points and the confidence threshold as sliding cursors (blue is the lower threshold and red, the upper). The user is able to drag either threshold using the mouse thereby including or rejecting individual samples.

Results table

Each sample is categorized in the results table as positive, negative or undetermined for each reporter based on:

(+) Positive: sample value > upper threshold.

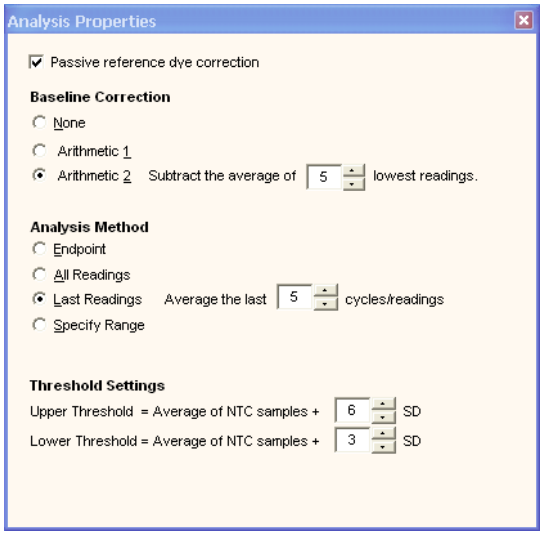
(-) Negative: sample value < lower threshold.

(?) Undetermined: sample value is between lower and upper thresholds.

A **Score** is given if one of the dyes is designated an IPC. In the example below, the FAM reactions are designated IPCs and should appear positive in each well unless there is a fault with the PCR e.g. inhibitor present.

No	Well ID	Sample ID	FAM	Yakima Yellow	Score
5	B10	Unknown 5	+	?	Retest
5	B11	Unknown 5	+	-	Negative
5	B12	Unknown 5	+	-	Negative
6	C1	Unknown 6	+	+	Positive
6	C2	Unknown 6	+	+	Positive
6	C3	Unknown 6	+	+	Positive
7	C4	Unknown 7	+	+	Positive
7	C5	Unknown 7	+	+	Positive
7	C6	Unknown 7	+	+	Positive

4.8.3.1 Viewing and changing the parameters



Click the **PAR** button next to one of the graphs to bring up the analysis settings for plus-minus scoring. If any settings are changed, the data will be recalculated and the graphs and results table updated accordingly.

The settings or analysis method can also be changed by accessing the **Analysis Selection** box from the Results Editor main page.

If a PRD was assigned in the dye usage box, then the PRD correction options can be accessed by clicking the PAR icon next to the raw data graph.

If the raw data graph is not shown click the raw data graph icon:

4.8.4 PrimeQ Report

The report options can be changed from within the report tab of the Results Editor. Click on the **Report Options** icon, which will bring up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click **Done** to finish.

4.8.5 Quick guide to plus-minus scoring analysis

1. In the Analysis Selection box, highlight the stage on which plus-minus scoring is to be performed and click **Edit**.
2. In the Analysis Wizard Selection box, select **Plus-minus scoring** from the drop-down menu and assign a use next to the appropriate dye(s) name. Click Next.
3. If a PRD was assigned, the PRD correction screen will be displayed. Choose whether to perform a PRD correction on the data and click **Next**.

4. Baseline correction: Select which method to use (proportional is not an option in plus-minus scoring) and click **Next**.
5. Choose a plus-minus analysis method and specify a range of readings if necessary. Click **Next**.
6. Set upper and lower confidence thresholds. Click **Next**.
7. Report options: Decide how to display the data in the PrimeQ report. Plus-minus score table is the default setting but choose to display in graphical format. Click **Next**.
8. Summary of analysis. Click **Finish** to return to the Experiment or Results Editor main screen.

4.9 Analysis method: Allelic discrimination

Allelic discrimination is a technique that is able to detect single base pair differences. It is used to discriminate between genotypes, mutations and polymorphisms within or between samples.

Conventional approaches to genotyping and mutation have traditionally relied on a three-stage process: amplification (e.g. PCR), discrimination (e.g. restriction digest) and detection (often radioactive), which are often time-consuming, complicated and costly. Allelic discrimination on the PrimeQ real-time PCR system allows these stages to be performed automatically within the reaction tube, thereby providing an assay which is rapid, reliable and cost-effective in terms of both reagents and time.

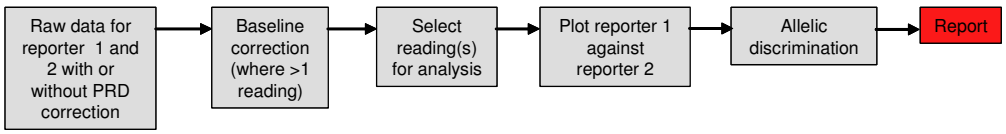
Principle

A single base mismatch within the region complementary to the probe will reduce its stability and therefore the Tm. The most common assay to use is the hydrolysis probe assay using dual-labelled probes. In this assay, PCR master mix, primers and fluorogenic probes with different labels constructed for the two allele sequences (located between the primer sites) are run in a thermal cycler with the unknown samples. During the PCR, the fluorescent probes anneal specifically to complementary sequences between the forward and reverse primers sites on the template DNA. During the extension cycle of the PCR, *Taq* DNA polymerase cleaves the hybridized probe and due to separation of the reporter dye from the quenchers, an increase in fluorescence is seen. When the allele is placed in the centre of the probe it has the maximum effect on the probe-template stability. Thermodynamically it is far more favourable for only the matching probe to bind to the template than the probe containing the mismatch.

4.9.1 Assay requirements

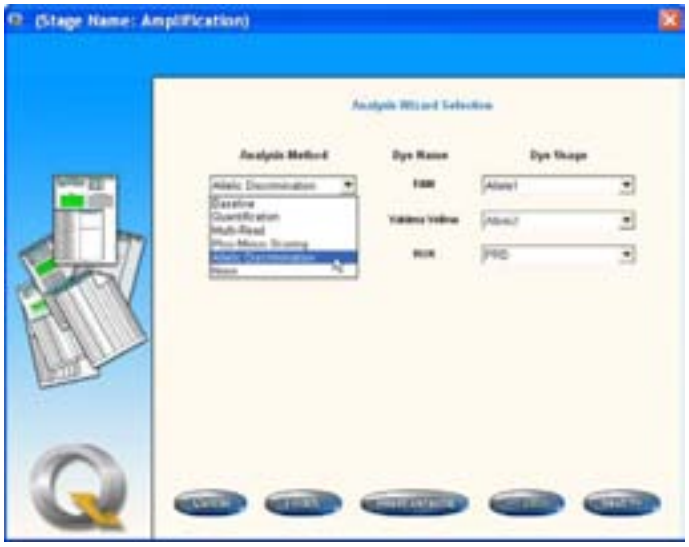
- Need at least two reporter dyes.
- Need at least one reading.
- Need a dye defined as PRD for PRD correction tab to be visible.
- Need at least two readings for baseline correction and analysis methods tabs to be shown.
- Positive control (POS) for each allele

4.9.2 Setup



The setup of an allelic discrimination assay in Quansoft follows a similar path to plus-minus scoring. Options for baseline correction are chosen and then a choice provided as to which readings to analyse. The fluorescence level of the reporters is then plotted against each other on a scatter graph.

- In the **Analysis Selection** box, highlight the stage name for the analysis to be applied, click **Edit** and the Analysis Wizard will launch.
- Choose **Allelic Discrimination** from the drop-down menu in the Analysis Method Selection box.
- In the **Dye Usage** box, assign one reporter as Allele 1 and the other reporter as Allele 2. If the user attempts to proceed with only one dye assigned then a message will be displayed telling the user that two dyes must be assigned for this analysis method to be performed.



- Click **Next** and the Allelic Discrimination Wizard will launch.

4.9.2.1 Baseline correction

The baseline correction methods are the same as for plus-minus scoring in that the data should be kinetic (i.e. > 1 reading) and that the Proportional baseline correction method is unavailable.

- Choose the appropriate method and click **Next**.

4.9.2.2 Allele scoring method

The purpose of this part of the analysis setup is to define which readings should be used for allele scoring. The options displayed are the same methods used as in plus-minus scoring.

- **End-point (default):** Uses the last reading only (we recommend that >1 reading is used for accuracy).
- **All readings:** Averages all readings in the stage
- **Last readings**: Averages a user-specified number of last readings (the default is 5, or 1 if there are less than 5 readings)
- **Specify range:** Specify a range of readings to be averaged that best suit the data (the default is the last reading).

4.9.2.3 Reporter selection

The fluorescence of the first reporter is plotted against the second and displayed on a scatter graph.

4.9.2.4 Report Options and Summary

- Click **Next** to lead through to the **Report Options** screen. The default is for an allelic discrimination graph and a table of results.
- Click **Next** to view a **Summary** of the setup.

- Click **Back** to change any settings or **Cancel** to abort the procedure.
- Click **Finish** to complete the set up.

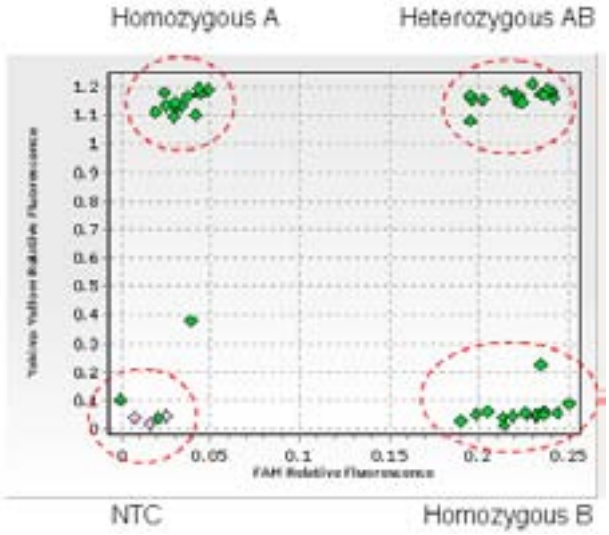
4.9.3 Viewing the results

During the run, the real-time collection of data can be monitored on the Run screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.

4.9.3.1 Allelic discrimination scatter graph

Particular to the allelic discrimination method is the display of a scatter plot representing the fluorescence of one allele plotted against the other. The software plots the results of the run on a scatter plot of allele 1 vs. allele 2 (axes defined by the user in Reporter Selection) with each well of the 96-well plate represented by a point on the graph.



The scatter graph shows the fluorescence values of each reporter in a given well plotted against each other

- If the scatter graph is not displayed, click on the icon to bring up the data:

- To swap the axes over, return to the **Reporter Selection** screen in the Allelic Discrimination Wizard.

Four distinct clusters can be seen representing the no template control (NTC) and three possible genotypes: homozygous A (AA), or B (BB) and heterozygous (AB).

Homozygotes will show increased fluorescence along one axis of the plot (depending on which reporter they contain), while the heterozygotes appear in the centre of the plot as they contain

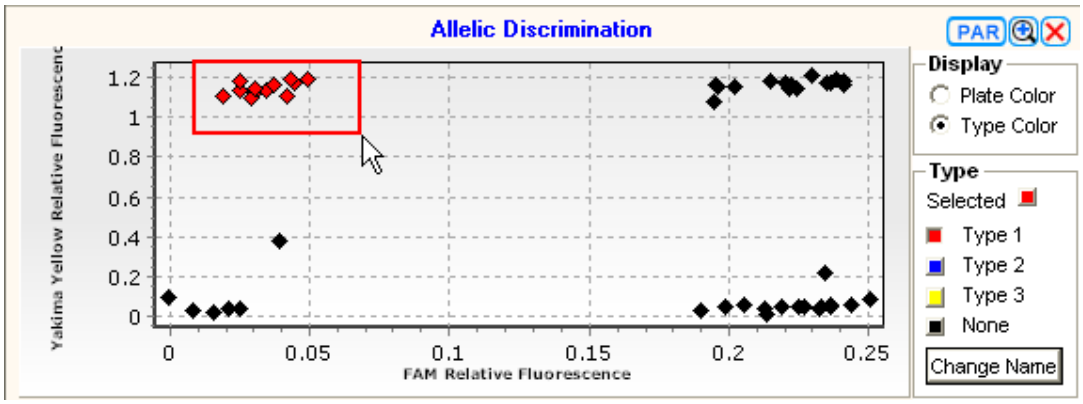
copies of both alleles and therefore produce fluorescence with both dyes. Samples that do not cluster tightly may contain rare sequence variations, sequence duplications or the PCR may not have been optimal.

Assigning Genotypes

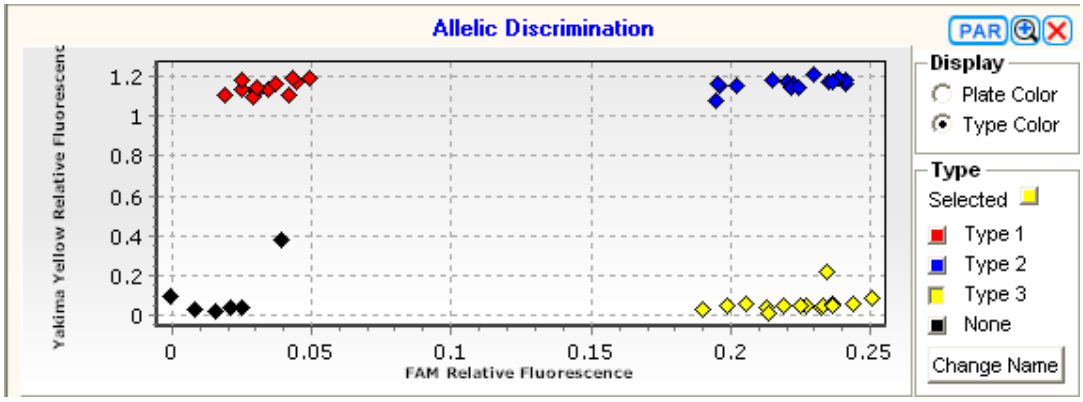
The user can define clusters of points within the plot that represent genotypic segregation within the samples; the scoring of each sample is then presented in the Results table.

- Click **Type Colour** in the **Display** box.
- Select one of the coloured **Type** tiles in the **Type** box.
- To assign a sample to that type, either click on individual sample points or use the mouse to draw a box around the cluster. To enhance visibility, maximize the scatter graph by clicking on the zoom icon in the top-right corner of the pane.

The selected data points will change colour to that of the selected Type tile.



- Repeat for the remaining data until all genotypes have been defined.



Each type can be given a name which will then appear in the results table.

- Click on **Change name** in the **Type** box and an **Allele Type** box will open.
- Type in the required names and click **OK** to accept.

Results table

For each well a fluorescence value for each reporter dye is displayed in the Results table together with its allele type determined from the typing assigned in the scatter graph. To change the order of the axes, enter the analysis setup using the **PAR** button or start the Allelic Discrimination Wizard from the Analysis Selection box on the Results Editor main screen. In **Reporter selection** change the order of alleles to compare.

No	Well ID	Sample ID	FAM	Yakima Y...	Type
1	A4	Unknown 1	0.03	1.13	Homozygous A
1	A5	Unknown 1	0.04	1.17	Homozygous A
1	A6	Unknown 1	0.02	1.13	Homozygous A
2	A7	Unknown 2	0.19	0.03	Homozygous B
2	A8	Unknown 2	0.21	0.06	Homozygous B
2	A9	Unknown 2	0.2	0.05	Homozygous B
3	A10	Unknown 3	0.2	1.15	Heterozygous AB
3	A11	Unknown 3	0.2	1.15	Heterozygous AB
3	A12	Unknown 3	0.21	1.19	Heterozygous AB
4	B1	Unknown 4	0.02	1.18	Homozygous A
4	B2	Unknown 4	0.03	1.13	Homozygous A
4	B3	Unknown 4	0.04	1.1	Homozygous A

4.9.3.2 Viewing and changing the parameters

Click the **PAR** button next to one of the graphs to bring up the analysis settings for allelic discrimination setup. If any settings are changed, the data will be recalculated and the graphs and results table updated accordingly.

The settings or analysis method can also be changed by accessing the Analysis Selection box from the Results Editor main page.

4.9.4 PrimeQ Report

The report options can be changed from within the report tab of the Results Editor. Click on the **Report Options** icon, which will bring up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click **Done** to finish.

4.9.5 Quick guide to allelic discrimination analysis

1. In the Experiment or Results Editor Analysis Selection box, highlight the stage on which allelic discrimination is to be performed and click **Edit**.

2. In the Analysis Wizard Selection box, choose Allelic Discrimination from the drop-down menu and assign a use next to the appropriate dye(s) name. Click **Next**.
3. Baseline correction: Choose which method to use (proportional is not an option in allelic discrimination) and click **Next**.
4. Select an allele scoring method. Click **Next**.
5. Select which reporter to compare to which. The fluorescence readings of the first reporter will be plotted against those of the second and displayed as a scatter graph. Click **Next**.
6. Report options: Decide which data should be displayed in the PrimeQ report. A table of results is the default setting but choose to display the data in a graphical format if required. Click **Next**.
7. Summary of analysis. Click **Finish** to return to the Experiment or Results Editor main screen.

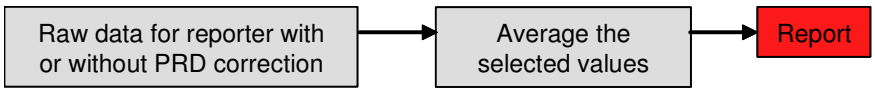
4.10 Analysis method: Multi-read

This analysis method averages all or a selected range of values in the stage for a given well. Multi-read is a useful tool in end-point analysis; end-point fluorescence values can be compared to Cq values obtained from quantification analysis of the amplification stage and used to aid primer optimization experiments. Alternatively, Multi-Read allows PrimeQ to be used simply as a plate reader.

4.10.1 Assay requirements

- Need at least one reporter.
- Need more than one reading.
- Need a dye defined as PRD for the PRD correction to be enabled.

4.10.2 Setup



- In the Analysis Selection box, highlight the stage name for analysis to be applied and click **Edit**. The Analysis Wizard will launch.
- Choose **Multi-Read** from the drop-down menu in the Analysis Wizard Selection box and assign dyes usage(s).
- Click **Next** and the Multi-Read Wizard will launch.

If a PRD was assigned in the Dye Usage Box then the next screen provides the option to apply PRD correction to the data.

- Click **Next**.

4.10.2.1 Multi-read selection method

The next screen allows the user to choose which readings are to be averaged.

- **End-point:** Uses the last reading only (we recommend that >1 reading is used for accuracy).
- **All readings (default):** Averages all readings in the stage
- **Last readings**: Averages a user-specified number of last readings (the default is 5, or 1 if there are less than 5 readings)
- **Specify range:** Specify a range of readings to be averaged that best suit the data (the default is the last reading).

4.10.2.2 Report Options and Summary

- Click **Next** to lead through to the **Report Options** screen. This allows the user to choose whether to include the multi-read table in the report. Check the box to select.
- Click **Next** to view a **Summary** of the setup.

- Click **Back** to change any settings or **Cancel** to abort the procedure.
- Click **Finish** to complete the set up.

4.10.3 Viewing the results

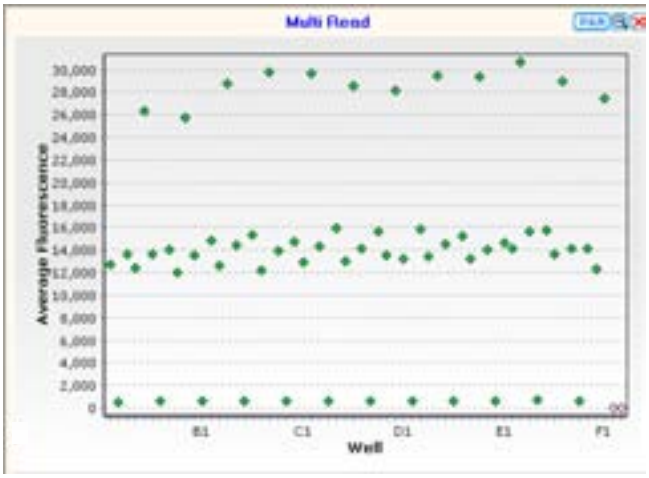
During the run, the real-time collection of data can be monitored on the Run Screen. The plate layout shows the fluorescence curve on a per-well basis and the temperature profile plot indicates how far the run has progressed.

When the run has completed, results can be viewed in the Results Editor with data from each stage of the run located under its own tab.

Multi-read graph

The graph displays the average readings for each well as colour-coded points.

If the multi-read graph is not displayed, simply click on the Multi-read icon to bring up the data:



- Clicking a well(s) in the plate layout will highlight the selected well on the multi-read graph.

Results table

The data is displayed with the sample/well ID and an average of the selected readings in each well. This value is plotted on the multi-read graph.

No	Well ID	Sample Name	Average/Well	Average/Rep...
1	A1	Unknown 1	12756.6	
1	A2	Unknown 1	546.8	6651.7
2	A3	Unknown 2	13604.8	
2	A4	Unknown 2	12411	13007.9
3	A5	Unknown 3	26368	
3	A6	Unknown 3	13632.8	20000.4
4	A7	Unknown 4	581.6	
4	A8	Unknown 4	14019.2	7300.4
5	A9	Unknown 5	12025.8	
5	A10	Unknown 5	25723.8	18874.8
6	A11	Unknown 6	13473.8	
6	A12	Unknown 6	603	7038.4
7	B1	Unknown 7	14789.4	
7	B2	Unknown 7	12581.2	13685.3
8	B3	Unknown 8	28775.2	

4.10.3.1 Viewing and changing the parameters

Click the **PAR** button next to the Multi-Read graph to bring up the analysis settings. If any of the settings are changed, the data will be recalculated and the graphs and results table updated accordingly.

The settings or analysis method can also be changed by accessing the Analysis Selection box from the Results Editor main page.

If a PRD was assigned in the dye usage box, then the PRD correction options can be accessed by clicking on the PAR icon next to the raw data graph.

If the raw data graph is not shown click the raw data graph icon:

4.10.4 PrimeQ Report

The report options can be changed from within the report tab of the Results Editor. Click on the **Report Options** icon, which will bring up the Report Options box. Tabs will display the report options relevant for each stage. Change as appropriate and click **Done** to finish.

4.10.5 Quick guide to multi-read analysis

1. In the Experiment or Results Editor Analysis Selection box, highlight the stage on which multi-read analysis is to be performed and click **Edit**.
2. In the Analysis Wizard Selection box, choose **Multi-Read** from the drop-down menu and assign a use next to the appropriate dye(s) name. Click **Next**.
3. Passive reference dye correction: If a PRD was assigned in the dye usage screen, options for correction will be displayed. Click **Next**.
4. Choose a Multi-Read analysis method and select a range of readings if required. Click Next.
5. Report options: The user can choose to send the multi-read table to the report by selecting this option. Click **Next**.
6. Summary of analysis. Click **Finish** to return to the Experiment or Results Editor main screen.

4.11 Multiplex assays

Multiplex assays (or multi-colour detection) combine the use of two or more different reporters in the same well. PrimeQ's cartridge carousel can house up to four pairs of excitation and emission filters, and as such, is capable of detecting up to four different reporters.

Multiplexing is suitable for all analysis methods as long as different reporters are used with different emission spectra and that if more than one PCR is occurring in the same well, the reactions do not compete. Furthermore, if the results are to be compared, the reactions should also have similar efficiencies. These are recommended to be in the range of 1.95 to 2.05. Multiplex assays are useful when analysing against internal controls, interpreting duplex PCR runs or in mutation analysis as detailed in **Allelic discrimination** (section 4.9).

Multiplexing is a cost-effective approach, bringing savings in terms of reagents, sample and block space. The method also helps when comparing results, as reactions are subjected to the same PCR conditions. An associated disadvantage however is that optimizing a multiplex assay is frequently a time-consuming process.

Example applications include:

Relative quantification

This analysis method compares the concentration of two reporters in a single well. The experiment is designed in the same way as the basic quantification assay but allows the comparison of two different reporters in the same well. This cuts down on reagents and allows more reliable comparison between the two targets as reaction conditions are guaranteed to be the same and the amount of starting material is the same. This approach requires the use of standards.

Allelic discrimination

Capable of detecting single nucleotide differences, this analysis method can only be used if there are two reporters. Probes specific for allele 1 and allele 2 are labelled with different reporter dyes and as mismatches between a probe and target will reduce the efficiency of the hybridization, this will be reflected in the relative fluorescent signal post-PCR. Comparing the relative fluorescence of each reporter will indicate whether the DNA sample is homozygote or heterozygote for allele 1 and 2 (see section 4.9.3.1).

Internal controls

A second set of primers and reporter labelled probe are used to amplify a second DNA template which is spiked into each PCR reaction. The control serves as a performance indicator for the PCR and is particularly useful in plus-minus scoring to guard against negative calls when a PCR has simply failed, or there is an inhibitor in the sample.

4.11.1 Multiplex setup

To perform a multiplex assay, two or more reporters must be defined in the Program.

- Define the thermal cycling parameters as detailed in section 3.4.3.
- Add a dye read to the required step as detailed in section 3.4.3.5.
- Add further reads to the step by clicking on the **Add Read** button. The reads will appear as tabs in the step settings box.

Refer to the relevant sections in Chapter 4 for detailed information on the analysis setup for individual methods.

Having assigned multiple reporters in the program setup, these will now be available when setting up the analysis method.

Note that the setup and analysis parameters will be consistent between dyes; if the user wishes to analyse dyes differently, then the analysis settings must be changed post-run from the Result Editor.

4.11.1.1 Viewing the results

- **Dye to View:** Use this option to view the data for the individual reporters.
- **Results table:** Results for only the dye currently being viewed will be listed in the results table unless an analysis method has been selected where more than one reporter is included in the calculation e.g. relative quantification, relative quantification cycles, allelic discrimination etc.

4.11.1.2 Viewing and changing the parameters

Analysis settings can easily be changed post-run, which can be useful in multiplexing experiments if the user wishes to treat one reporter differently to another.

To change the parameters, click the **PAR** icon button next to the graphs to bring up the settings box appropriate to the analysis method. If any settings are changed, the data will be recalculated and the graphs and results table updated accordingly.

The settings or analysis method can also be changed by accessing the **Analysis Selection** box from the Results Editor main screen.

5 Technical information

Technical information on PrimeQ

About this chapter

This chapter provides all the technical information you may need to know about your PrimeQ real-time PCR system. It covers everything from spare parts to recommended consumables and maintenance routines.

5.1 Operator maintenance

NOTE THAT THIS EQUIPMENT SHOULD ONLY BE DISMANTLED BY PROPERLY TRAINED PERSONNEL.

REMOVING THE CASE EXPOSES POTENTIALLY LETHAL MAINS VOLTAGES.

THERE ARE NO OPERATOR MAINTAINABLE PARTS WITHIN THE EQUIPMENT.

In the unlikely event that you experience any problems with your unit which cannot easily be remedied, you should contact your supplier and return the unit as necessary. Please include any details of the fault observed and remember to return the unit in its original packing. Techne accept no responsibility for damage to units which are not properly packed for shipping: if in doubt, contact your supplier giving the full serial number of the unit and the firmware version number (shown on the LCD screen when the unit is first switched on). See the De-contamination Certificate supplied with your unit.

5.1.1 Cleaning PrimeQ

Before cleaning your unit ALWAYS disconnect from the power supply and allow to cool below 50°C.

The thermal block, including wells and flat surfaces, should be cleaned regularly to ensure optimum heat transfer to the samples. Always clean the block if there has been a spillage. Use a cloth or cotton buds dipped in a fresh, 50:50 water/isopropanol solution and make sure that no deposits are left in the wells. To remove the block for more detailed cleaning, see section 5.2.

The unit can be cleaned by wiping with a damp soapy cloth. Care should be exercised to prevent water from running inside the unit. A cloth dipped in ethanol, methanol or formaldehyde can also be used. No part of the case or cover should be immersed in the solvents. Do not use aggressive solvents such as acetone or abrasive cleaners.

In the case of radioactive spillages, Techne recommend that you use a proprietary cleaning agent. Carefully follow the cleaning agent manufacturer's instructions. The thermal block is made of aluminium. Therefore, an agent such as Neutracon (from Decon Laboratories Ltd.), suitable for nonferrous metals should be used but remember other parts of the unit are made of ferrous materials and may be damaged by spillage onto them.

Before using any cleaning or decontamination method except those recommended here, the responsible body should check with Techne that the proposed method will not damage the equipment.

5.1.2 Fuses

Your unit is protected by two fuses. These should only be changed by suitably qualified personnel.

If the fuses blow persistently, a serious fault is indicated and you may need to return the unit to your supplier for repair.

If the display on the front panel is not lit, one of the two fuses may have blown. Check that there is no external cause, such as a faulty plug or lead. Check both fuses and replace the faulty fuse with a new one of the correct value (fuse values are given on the label next to the power inlet.) Note that fuses should only be replaced by a qualified electrician.

The holder for the two fuses is built into the power input socket. First remove the power cord, and then gently lever the fuse drawer open with a flat-bladed screwdriver or similar tool.

Each fuse can be removed by using the screwdriver as a lever.

Exchange the faulty fuse in the fuse holder for a working fuse of the correct value. Finally, replace the fuse drawer in the fuse compartment and push the drawer shut.

5.1.3 Insulation Testing

This equipment is fitted with RFI suppression circuitry. Any check of the electrical insulation by means of high voltage dielectric testing must be carried out using only a DC voltage.

This unit contains semiconductor components which may be damaged by electric field effects.

5.1.4 Mantenimiento

ESTE APARATO DEBE SER DESMONTADO SOLO Y EXCLUSIVAMENTE POR PERSONAL DEBIDAMENTE CAPACITADO.

EL RETIRAR LOS PANELES LATERALES, FRONTALES O TRASEROS SUPONE DEJAR AL DESCUBIERTO TENSION DE LA RED PELIGROSA.

EL EQUIPO NO CONSTA DE NINGUNA PIEZA DE CUYO MANTENIMIENTO SE PUEDA ENCARGAR EL USUARIO.

En el caso improbable de que experimentara algún problema con su aparato que no pudiera resolver con facilidad, debería ponerse en contacto con su proveedor y devolverlo si fuera necesario. Indique de forma detallada todos los defectos que haya notado y devuelva el equipo en su embalaje original. Techne no aceptará responsabilidad alguna por daños causados en equipos que no estuvieran debidamente embalados para su envío; si tuviera alguna duda, póngase en contacto con su proveedor. Sírvase consultar el Certificado de Descontaminación suministrado con su aparato.

1. Limpieza

Antes de limpiar su aparato, desconéctelo SIEMPRE de la fuente de alimentación y permita que se enfríe por debajo de los 50ºC.

Este aparato se puede limpiar pasándole un paño húmedo enjabonado. Hágalo con cuidado parae evitar que caiga agua dentro del mismo. No utilice limpiadores abrasivos.

2. Fusibles

Su aparato está protegido por dos fusibles. Sólo deben cambiarlos personal debidamente capacitado.

Si los fusibles se fundieran repetidamente, esto indicaría una avería grave y puede que tuviera que devolverle el aparato a su proveedor para su reparación.

5.1.5 Entretien utilisateur

IMPORTANT : CET APPAREIL NE PEUT ETRE DEMONTE QUE PAR DU PERSONNEL QUALIFIE.

LORSQUE LES PANNEAUX AVANT, ARRIERE ET LATERAUX SONT DEMONTES, L'OPERATEUR EST EXPOSE A DES TENSIONS QUI PEUVENT ETRE MORTELLES.

CET APPAREIL NE CONTIENT AUCUN ELEMENT QUI DEMANDE UN ENTRETIEN DE LA PART DE L'UTILISATEUR.

Dans le cas peu probable où votre appareil présente un défaut de fonctionnement auquel il est difficile de remédier, il est alors préférable de contacter votre fournisseur et, le cas échéant, de renvoyer le matériel. Veuillez inclure une description détaillée du problème constaté et retourner l'appareil dans son emballage d'origine. Techne ne sera pas tenu responsable des dommages subis par tout appareil dont l'emballage est inadéquat pour le transport. Pour plus de sûreté, contactez votre fournisseur. Voir le certificat de décontamination livré avec le produit.

1. Nettoyage

Avant de nettoyer l'appareil, assurez-vous TOUJOURS que le câble d'alimentation est déconnecté et laissez la température redescendre en dessous de 50ºC.

Utilisez un chiffon imprégné d'eau savonneuse pour nettoyer l'appareil. Veillez à ne pas introduire d'eau dans l'appareil. N'utilisez pas de produits abrasifs.

2. Fusibles

La protection de l’appareil est assurée par deux fusibles dont le remplacement ne peut être effectué que par un personnel qualifié.

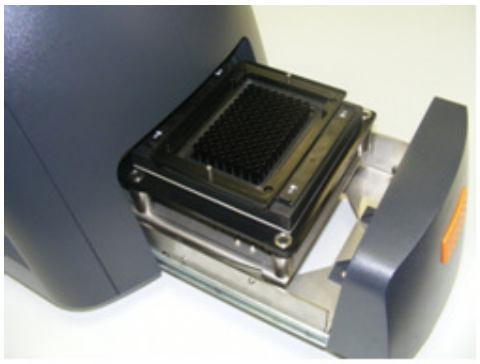
Si les fusibles sautent sans arrêt, il s’agit d’un problème sérieux. Nous vous conseillons dans ce cas de prendre contact avec votre fournisseur pour réparation

5.2 Block Access

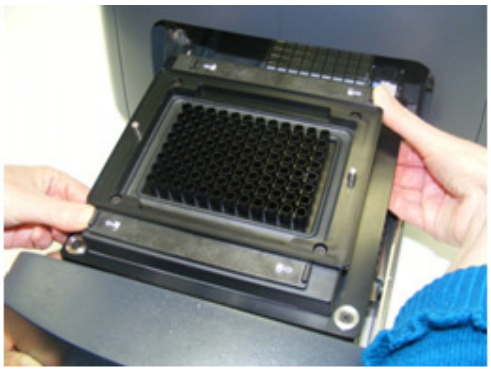
The block can be removed for more thorough cleaning, or maintenance.

Ensure that the instrument has been idle long enough for the block and heated lid to have cooled down to a safe temperature to handle.

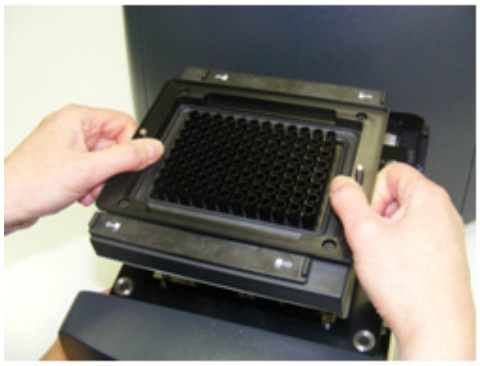
SWITCH OFF THE PRIMEQ UNIT AND UNPLUG THE POWER CABLE BEFORE REMOVING THE BLOCK.



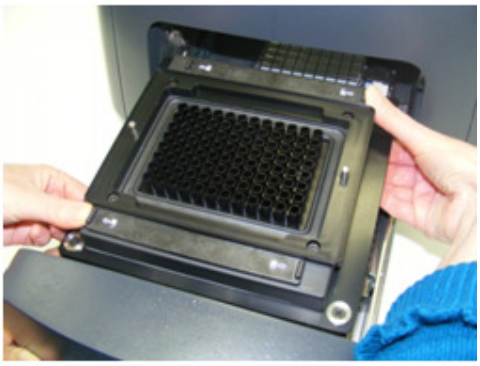
1. Open the drawer



2. Slide the quick release handles in the UNLOCK direction (arrows)



3. Lift the block from its mounting



4. After replacing the block LOCK the handles

Unlock the release handles (slide towards open padlock position) and lift the block assembly from the unit. Never lift or carry the block by one side only, always use both sides or support the block from underneath. When you have cleaned the block, carefully place the block back in position. Ensure the block is fully engaged by pushing all four corners of the block down into the carrier. Then slide the quick release handles in the opposite direction (closed padlock position) to lock the block assembly in position.

The thermal blocks are internally calibrated so when replaced, temperature performance is unchanged and recalibration is not required.

The PrimeQ unit is guaranteed for a period of 2 years from the date of purchase.

5.3 User responsibilities

- To use the instrument in such a manner as to minimize interference with the optical system. The heat-sealed film over the top of the plate must be clean and dry when the plate is loaded as the optical components will make contact with this surface. The film must be free of foreign bodies, creases, folds or finger marks.
- To install the required filter sets and ensure they are correctly positioned and secured (see instructions for fitting in section 1.16.3). The user must replace filters when degraded with age.
- To change the thermal block when required.
- To keep the instrument clean and air inlets and outlets free from obstruction.
- To load the correct type of sample plate.

5.3.1 LED

The long-life LED used in PrimeQ is extremely durable and should last the life of the instrument.

In the unlikely event any issues arise with this item, please contact Techne for support.

5.3.2 Filters

The life of the emission and excitation filters is expected to be at least two years but is dependent upon the ambient temperature and humidity.

See chapter 1 for further details.

For part numbers see section 5.6.

The software is only able to detect whether a cartridge is present and not its specifications. These must be defined by the user via the application software. See section 1.16.4 for more information.

5.3.3 Replacing the fuses

Ensure that fuses are only replaced with the same type and rating as those currently in the instrument.

5.4 Consumables

For general purpose use, giving good sensitivity and resolution, Techne recommends the use of low profile 96-well clear plates. Black plates give the lowest background, and white plates, although they have the highest background, may be advantageous for some probe-based chemistries.

PrimeQ does not require a specific seal as long as the seal of choice is compatible with the unit's optical reads.

For further recommendations and questions regarding consumables, please contact PrimeQHelp@bibby-scientific.com or PrimeQHelp@techneusa.com.

5.5 Minimum computer requirements

A PC is not supplied with PrimeQ

The following are the recommended minimum PC specifications required for running PrimeQ:

CPU:	Core 2 Duo or equivalent
Memory:	2Gb DDR RAM as a minimum
Storage:	40Gb DMA hard drive
Display:	1024 x 768 resolution minimum, 17 inch digital monitor recommended.
Drive:	DVD/CD-RW drive
Operating System:	Microsoft® Windows® XP Professional SP3 or later, Windows® Vista, Windows® 7
Connections:	USB
Also useful:	
Sound:	Built-in sound, video and LAN facilities
Internet:	Ethernet connection
Software:	Internet Explorer, Microsoft® Office

5.6 Accessories

The following accessories can be obtained from Techne or your Techne dealer. Please note that additional filters may be available upon request:

Part number	Description	Unit quantity
FC01	Filter cartridge for FAM multiplex	1
FC02	Filter cartridge for FAM/SYBR®	1
FC03	Filter cartridge for HEX/TET/JOE/VIC/Yakima Yellow	1
FC04	Filter cartridge for ROX	1
FC05	Filter cartridge for Cy5/Quasar® 670	1

For full details of recommended filter cartridges and dyes for multiplexing please contact Techne on PrimeQHelp@bibby-scientific.com or PrimeQHelp@techneusa.com.

5.7 Replacement parts

The following replacement parts can be obtained from Techne or your Techne dealer:

Part number	Description	Quantity required
HH179(S)	UK mains lead with plug	1
HH180(S)	EU mains lead with plug	1
7002705	US Power cord and 3-pin plug 120V	1
6501267	Fuse T5A, 200V to 230V supplies	2
6500324	Fuse T10A, 100V to 120V supplies	2
7002926	USB cable	1
PRIMEQ/B	Block 96 x 0.2ml well plate	1
5100492	Packaging and fittings for PrimeQ	1

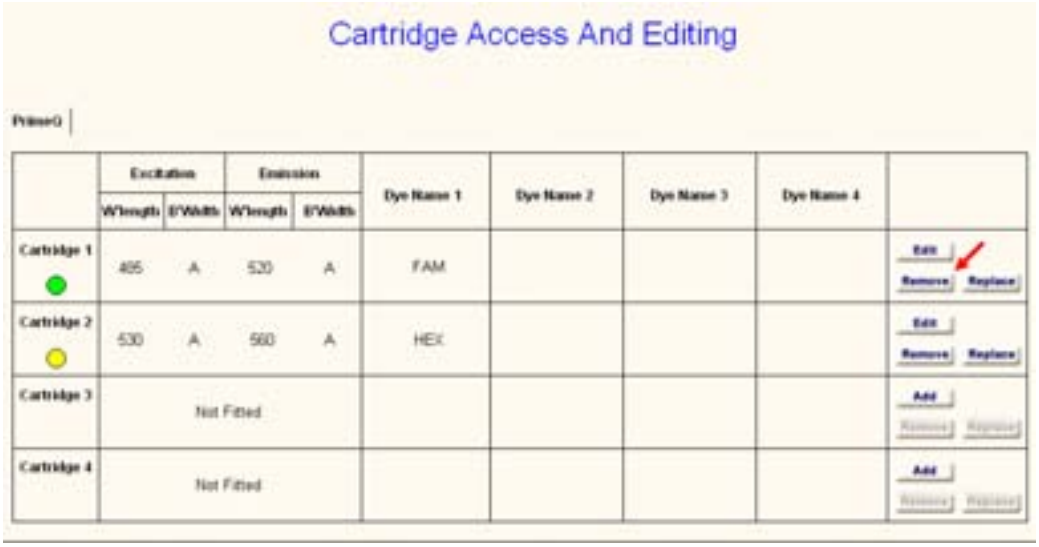
For full details of the Quansoft applications software (available free of charge) contact your local dealer, PrimeQHelp@bibby-scientifc.com or PrimeQHelp@techneusa.com.

5.8 Packing the PrimeQ instrument

5.8.1 Remove the filter cartridges

Before packing the PrimeQ for transit it is essential to remove the filter cartridges.

- In the Cartridge Access and Editing screen of the Filter Wizard, click **Remove** next to the appropriate filter.



- Follow the procedure as shown and click **Finish** to complete.

A message will appear informing the user to 'Please wait – checking cartridge status'.

If the removal was performed correctly, the Cartridge Access and Editing table will be updated to display **Not Fitted** next to the slot from which the cartridge was removed.

- Repeat for each filter cartridge until all have been removed.

- Wrap each filter in the tissue paper and place in the bag with the silica gel sachet. Replace in the filter cartridge storage box.

There is a space in the PrimeQ carton for the cartridge box, between the foam instrument surrounds.

5.8.2 Remove the block

To prevent damage to the drawer during transit, the block must be removed prior to packing the instrument, and packed in its own transit box for inclusion in the crate.

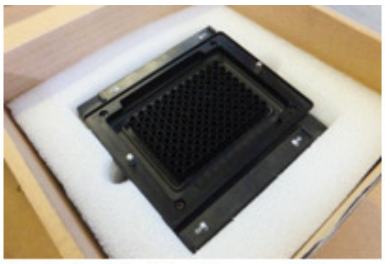
SWITCH OFF AND UNPLUG THE POWER CABLE BEFORE REMOVING THE BLOCK.

- Follow the directions given in section 5.2 for removing the block.

5.8.3 Packing the instrument

After block removal, ensure that the drawer is half open and wedged with the foam piece provided when the instrument is placed in the carton. This prevents the drawer from moving against its latch during transit, which could cause wear.

- **Pack the block and filters; wedge the drawer:**



Pack the block in the transit box.

Place the filters in the storage box.

Wedge the draw open using the packing foam piece.

- **Bag the PrimeQ and place into the carton base:**

The carton has a shaped base. Wrap the PrimeQ in the anti-static bag and place in the base of the carton.

- **Add foam retainers and the block and filter boxes:**

Fit the foam retainers around the PrimeQ.

The block and filter boxes fit into the front of the carton where the foam surround is cut away.

Check-list

Have you:

1. Removed the filters?
2. Removed the block?
3. Used the anti-static bag?
4. Packed the unit correctly?
5. Packed the block?
6. Packed all the accessories including the filter cartridges?

5.9 Packaging

On all correspondence, please quote the serial number in full and/or the Sales Order Number.

Any instrument requiring service under the guarantee should be taken to the supplier through whom it was purchased, or, in the case of difficulty, it should be carefully packed in its original packing and consigned, carriage paid, to us. Techne takes no responsibility for returned goods damaged in transit. If you do not have the original packing you can purchase a new pack from your dealer, part number 5100492.

5.9.1 Returns authorization number

Returned goods will not be processed without a Returns Authorization Number.

Call the Service Department +44 (0)1785 810475 for a number or email service@bibby-scientific.com.

In the US call +1 609 589 2560 or email service@techneusa.com.

Please write the Returns Number on the outside of any packing.

5.9.2 De-contamination certificate

Ensure that a copy of the Decontamination Certificate is visible on the outside of the packing. If you do not have your certificate a copy can be obtained from the Service Department as above.

6 Troubleshooting

Troubleshooting real-time PCR and PrimeQ

About this chapter

This chapter contains information required for troubleshooting the real-time PCR process, the control software and the instrument itself.

6.1 Troubleshooting

ISSUE	CAUSE	SOLUTION
No display on LCD screen.	No power to instrument.	Check power supply is connected.
	Fuse blown in plug.	Check fuse and change if appropriate.
No amplification.	Incorrect reaction conditions.	Optimize assay and run agarose gel to check PCR
	Reaction component not added.	Check correct reagents were added.
	Incorrect primer or probe sequence.	Redesign primers or probe.
	No template added to reaction or degraded template present.	Repeat assay.
	Reaction inhibitor present in template.	Ensure that the template is <10% of the assay volume. Purify template further.
	Sequence not present in sample.	To differentiate between true negative results and a false negative e.g. reaction component missing, always include a positive control.
	Dye has been exposed to light and bleached.	Do not expose components containing a dye to light. Do not freeze-thaw repeatedly.
	Wrong filter cartridge used during data collection.	Repeat assay with correct filter cartridge for the dye selected.
NTC shows amplification.	Amplicon or template contamination of one of the reagents.	Repeat assay with fresh reagents.
Large standard deviation between replicates.	Inaccurate pipetting.	Use a master mix for the reagents. Use a positive displacement pipette.
High fluorescence in one well.	Sample splashed on to plate seal.	Remove plate and inspect, if droplets on the film are easily visible, exclude the data from analysis.
	Extra master mix added in error.	Check the volume in the well.
Sudden drop in fluorescence.	Splash/droplet of master mix running down the well.	Centrifuge the plate briefly before thermal cycling.

ISSUE	CAUSE	SOLUTION
No Cq recorded for a sample.	No template added. No target in sample. Noise and crossing line set incorrectly.	Repeat assay. The default settings in Quansoft are 4 and 10 standard deviations respectively, adjust these through the parameter option or the Analysis Wizard.
Decreased volume in samples at end of a run.	Poor seal.	Ensure the sealing method is appropriate. Heat sealing is recommended.
Higher Cq than expected.	Fewer templates added to reaction. Template is degraded.	Increase amount of template added to reaction. Check sample integrity on an agarose gel or suitable bio analyser.
Lower Cq than expected.	More template added to the reaction. Template or amplicon contamination of one of the reagents.	Decrease amount of template added to reaction. Repeat assay with fresh reagents.
Small increase in fluorescence.	Concentration of dye is too low either because the dye has deteriorated or insufficient was added to the reaction. Poor PCR efficiency.	Protect reagents from light. Do not freeze-thaw repeatedly. Check optimization of dye concentration and add more if necessary. Optimize primer and probe concentrations. Check annealing temperatures of primers and probes. Optimize $MgCl_2$ concentrations (usually unnecessary if primers and probe were designed correctly).
More than one peak appears in the dissociation curve.	Two products of different length/GC content have been amplified; possible causes are miss-priming or pseudo-genes.	Check products on an agarose gel. Optimize annealing temperature of primers.
No specific peak appears in the dissociation curve, only primer-dimers.	Assay conditions preferentially amplify primer-dimers rather than the specific product.	Use a hot start enzyme. Increase annealing temperature of the primers to increase stringency.
Amplification plots have a flat slope.	Poor PCR efficiency.	Optimize assay conditions.

ISSUE	CAUSE	SOLUTION
Instrument cannot be seen in the pre-run screen or in the Cartridge Access and Editing screen.	Connection lost between instrument and computer.	Restart instrument and computer.
Fatal error displayed on LCD screen.	Instrument error.	Note the error message and contact your Techne Distributor.
Any other error which prevents a PCR run being initiated.	Instrument error.	Open Windows Explorer. Open C:\Program Files\Techne\PrimeQIM\Data Make a copy of the files in this folder – this will enable fault diagnosis by Techne.

6.2 Real-time PCR Glossary

Absolute quantification	A standard curve is used to quantify unknown samples by interpolating their quantity.
Allelic Discrimination	Technique used in genotyping to distinguish the presence or absence of two alleles using two different dye-labelled probes.
Amplicon	Segment of DNA generated by PCR.
Amplification plot	Graphical representation of fluorescence signal versus cycle number.
Background fluorescence	Fluorescence present in an assay before amplification which is due to inefficient quenching of the reporter dye, the inherent fluorescence of the reagents or consumables.
Baseline	The initial cycles of PCR in which there is little change in the fluorescence.
Calibrator	A specified cDNA or DNA sample to which all other samples in the experiment will be compared.
cDNA	Complementary DNA produced by reverse transcription of total or messenger RNA.
Cq	Quantitation cycle, the cycle number at which the fluorescence increases past a fixed threshold. PrimeQ has default values of 10 standard deviations above the noise for the threshold or Crossing Line.
Crossing Line	The best fit or threshold, significantly above the noise where the reaction efficiency is at its highest and most constant for each amplification plot.
Dissociation Curve	Graphical plot of fluorescence vs. increasing temperature. It is used for intercalating dyes to determine the temperature at which the double-stranded DNA is 50% separated.
Endogenous Control	An RNA or DNA template which is present in each sample and is amplified during the PCR via a second set of primers and probe e.g. Reference genes.
Exogenous Control	A characterized template which is spiked into each sample at a known concentration and is amplified during the PCR via a second set of primers and probe
IPC	Internal Positive Control: A control assay run together with the test samples as a multiplex. Often used in plus/minus scoring assays to ensure that a failed PCR is not mistaken for a negative result.
NTC	No template control: Sample which contains all the PCR reaction components except the DNA template.

PCR Efficiency	An efficient PCR assay shows a doubling in the specific product after every cycle: Reaction efficiency $E = 10^{-1/slope}$ ideal value is 2 The efficiency is affected by the length, GC content and secondary structure of the amplicon and sub-optimal reaction conditions.
PRD	Passive Reference Dye: Internal reference dye to which the reporter dye is normalized to correct for changes in volume and block variations.
R^2 value	Correlation coefficient which describes how well a line of Best Fit describes the relationship between two sets of data. A value of close to 1 defines good correlation whilst a value of 0 defines no correlation.
REF	Reference Gene. A gene expressed at an equivalent level in all the samples in the real-time PCR experiment. Sometimes known as a housekeeping gene.
Relative Quantification	Used to analyse changes in expression in a sample relative to another reference sample e.g. an untreated cell line.
Standard	Sample of known concentration used to construct a standard curve which is then used to extrapolate the quantity of an unknown sample.