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Eco™ Real-Time PCR System User Guide

FOR RESEARCH USE ONLY

ILLUMINA PROPRIETARY
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INTENDED USE: The Eco Real-Time PCR System is intended to support the Real-Time polymerase chain reaction (PCR) application needs of life science researchers. This includes gene expression quantification and analysis as well as genotyping by allelic discrimination or high-resolution melting. The system is able to support other applications and protocols as well. Eco features high-quality optical and thermal modules to provide optimal performance and data quality. The system includes data analysis software that is preloaded on a netbook computer and provided on a separate USB drive for installation on additional computers as needed. Additional accessories and consumables are provided or available for purchase to ensure the best user experience.

Use of the Eco for specific intended uses, such as polymerase chain reaction (PCR), Real-Time qPCR, or high-resolution melting (HRM) may require the user to obtain rights from third parties. It is solely the user's responsibility to obtain all rights necessary for the intended use of Eco.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s).

FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY.

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Overview

Chapter 1

Introduction

The Eco Real-Time PCR System offers life science researchers a full-featured real-time PCR system at an attractive price. Its features include:

- Four-color multiplexing
- High Resolution Melt (HRM)
- Fast PCR cycling: 40-cycle PCR in 40 minutes
- User-friendly, MIQE-compliant software

Eco's proprietary technologies provide excellent optical performance along with unmatched temperature control and thermal uniformity for a plate-based format (± 0.1°C).

Its robust optical system contains two sets of 48 LEDs, which provide excitation energy for a broad range of fluorophores, along with four emission filters and a CCD camera for detection, enabling multiplexing of up to four targets. Each instrument comes factory-calibrated for SYBR Green I dye, FAM, HEX, VIC, ROX, and Cy5.

Eco supports multiple applications, including gene expression quantification and analysis, and genotyping by allele discrimination or high-resolution melt (HRM). The system includes easy-to-use data analysis software preloaded on a netbook computer along with other accessories and consumables to provide the best user experience. The software is also provided on a USB drive so that it can be installed on additional computers for convenient access.

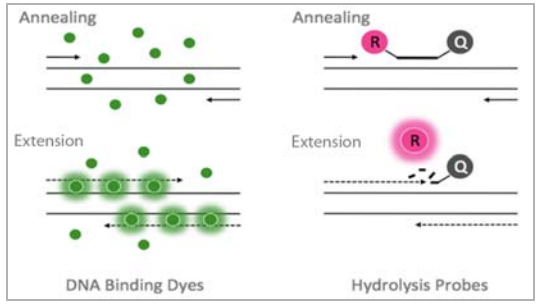
To order Eco materials and accessories, go to **https://my.illumina.com**. If you do not have an account yet, click **Create New User**.

Go to **http://www.illumina.com/ecoqpcr** for Eco resources, including tutorials, customer stories, and information about the many possible applications of Eco technology.

Real-Time PCR

Polymerase Chain Reaction (PCR) denotes the amplification of DNA templates catalyzed by DNA polymerase in the presence of primers, dNTPs, divalent cations (like Mg+2), and a buffer solution.

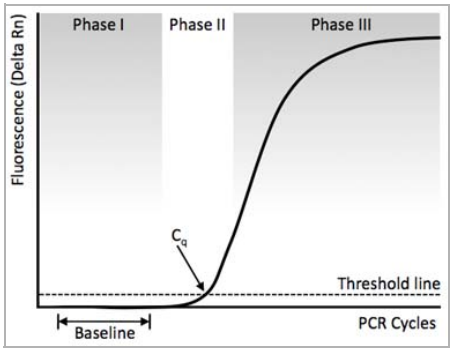
Figure 1 Main Real-Time PCR Chemistries



The ability to visualize and quantify the amplification of DNA as it occurs during PCR is called Real-Time PCR or Quantitative PCR (qPCR). This is made possible by the use of fluorescent chemistries, an optical system that can capture the emitted fluorescence at every PCR cycle, and software that can quantify the amplification.

The two most commonly used qPCR chemistries are DNA binding dyes and hydrolysis probes (Figure 1). DNA binding dyes fluoresce when bound to double-stranded DNA. Hydrolysis probes fluoresce when the reporter molecule is removed from its quencher molecule by the 5′ exonuclease activity of DNA polymerase.

Figure 2 The Three Phases of qPCR



Little fluorescence is generated during initial PCR cycles (Figure 2). Data from these early cycles define the baseline for the assay (Phase I). As fluorescence approaches the level of optical detection, the reaction reaches the exponential phase (Phase II), which is the region where the Cq is determined. Cq is the PCR cycle at which the fluorescent signal crosses the detection threshold level and is used for quantification. Finally, as reaction components are consumed and amplicons become abundant, the generation of additional fluorescent signal slows down and eventually reaches a reaction plateau (Phase III).

Resources

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Science 230: 1350–1354

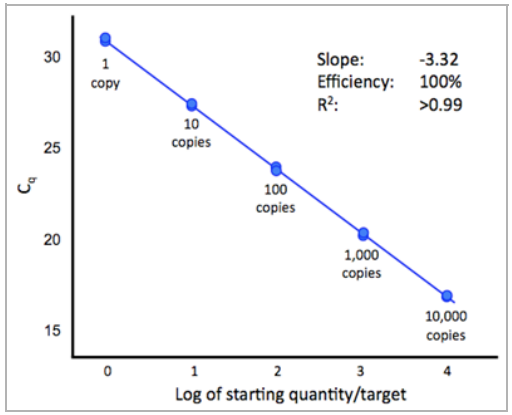
Higuchi R, Fockler G, Dollinger G, and Watson R (1993) Biotechnology (N.Y.) 11: 1026–1030

Absolute and Relative Quantification

The two primary methods used to quantify nucleic acids by qPCR are the absolute and relative quantification methods.

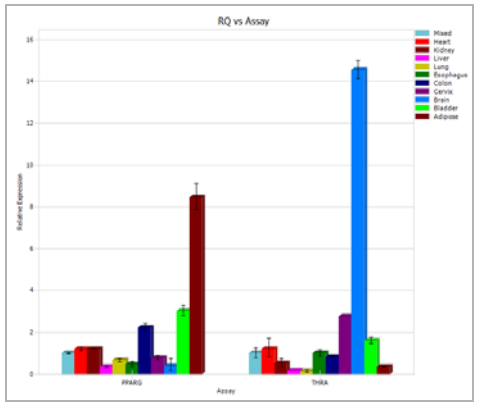
The absolute quantification method is based on a standard curve generated from serial dilution of a nucleic acid template of known concentration (Figure 3). Quantification of unknown samples is determined by interpolating the sample Cq from the standard curve. (Throughout the rest of this document, absolute quantification is referred to as a standard curve experiment.)

Figure 3 Five-Point (10-Fold) Standard Curve



The slope of the standard curve measures the efficiency of the assay ($E = 10^{[-1/slope]} - 1$). A slope outside the acceptable range (slope -3.1 to -3.6 and E value between 90 and 110%) typically indicates a problem with the template or assay design. The R^2 value, a measure of reaction performance, should be > 0.99 for the assay to accurately quantify unknown samples.

Figure 4 Relative Quantification Experiment

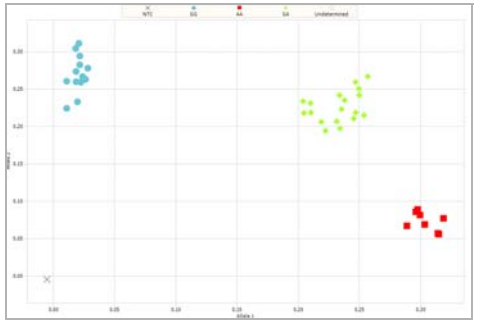


The relative quantification method measures the level of gene expression in a sample relative to the level of expression of the same gene in a reference sample. In addition, the level of expression of every gene in the assay is normalized to the expression of a reference gene.

The results (RQ value) obtained are expressed as relative levels (or fold change) in gene expression compared to the reference or control sample (Figure 4).

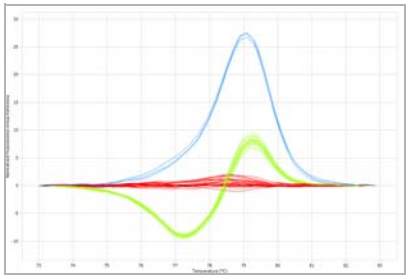
Allelic discrimination assays using hydrolysis probes provide a rapid and sensitive method to genotype samples. Two variants/alleles are interrogated at the same time (multiplex qPCR). Most frequently, one probe is labeled with a FAM dye and the other with a VIC dye. Samples with FAM signal and no VIC signal are homozygous for allele 1; samples with VIC signal and no FAM signal are homozygous for allele 2; and samples with both FAM and VIC signal are heterozygous (Figure 5).

Figure 5 Allelic Discrimination Scatter Plot



High Resolution Melt (HRM) enables the detection of almost any genetic variation (SNPs, mutations). Because HRM assays only require primers and a dye (no probes or DNA sequencing), the method is simpler and cheaper than traditional genotyping approaches. After the amplification phase, the amplicon is slowly heated until it melts. The melting temperature and profile are directly linked to the amplicon sequence.

Figure 6 HRM Difference Plot



HRM's power comes from the resolution of the sample's melt profile. It requires a high quality optical system and precise thermal uniformity. HRM PCR amplicons below 300 bp provide the best resolution. The shape of the resulting melting curves, which is sensitive to almost any genetic change, determines sample identity. To easily cluster equivalent samples, a reference curve (e.g. Wild Type) is subtracted from the other curves to generate a difference plot (Figure 6).

Resources

Livak KJ (1999) Allelic discrimination using fluorogenic probes and the 5′ nuclease assay. Genet Anal Biomol Eng 14: 143–149

POLAND server (http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html)

Wojdacz TK, Dobrovic A, Hansen LL (2008) Methylation-sensitive high-resolution melting. Nature Protocols 3(12): 1903–1908

Multiplexing Real-Time PCR

The simultaneous detection of multiple targets in a single reaction is called multiplexing. An advantage of multiplexing is that it conserves sample, allowing more data to be obtained from the same amount of material. Another advantage is that multiplexing permits the inclusion of an internal control reference assay for normalization purposes, significantly increasing data precision.

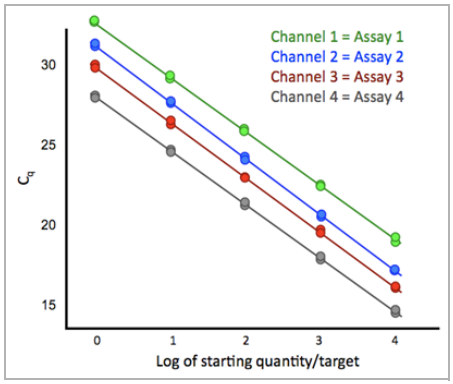
Validating a multiplex qPCR assay can be challenging. The advent of more advanced qPCR master mixes has significantly reduced the amount of optimization typically required, making multiplex qPCR a much more attractive alternative. Validation of assays using a standard curve is a must to ensure data accuracy.

Figure 7 Examples of Eco-Compatible Dyes

Channel 1 (λ = 505–545 nm)
SYBR Green[a], FAM[a]
Channel 2 (λ = 604–644 nm)
ROX[a], Texas Red
Channel 3 (λ = 562–596 nm)
HEX[a], JOE, TET, VIC[a]
Channel 4 (λ = 665–705 nm)
Cy5[a]

a. Factory-Calibrated Dyes

Figure 8 Standard Curves for Four Multiplexed Assays



The Eco Real-Time PCR System includes two excitation LED arrays (452-486 nm and 542-582 nm) and four filter channels (Figure 7), which enable detection of up to four separate targets in a single reaction (Figure 8).

Eco is factory-calibrated for certain dyes within each channel (marked in Figure 7), but also supports additional dyes that are excited and detected within the instrument specifications.

Setup

Chapter 2

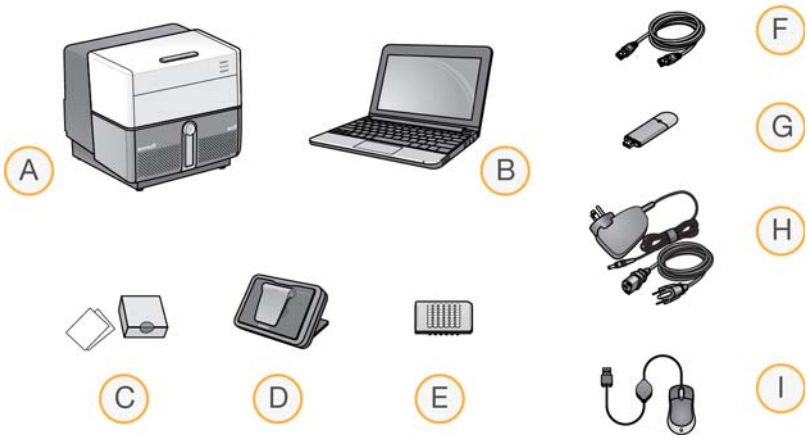
Unpack the Eco System

1 Remove the Dell netbook and accessories box from the crate.

2 Lift the Eco instrument out of the crate. Place it on a flat surface and remove the foam packaging.

NOTE
Keep the box and packaging in case of a return (repairs, for example.)

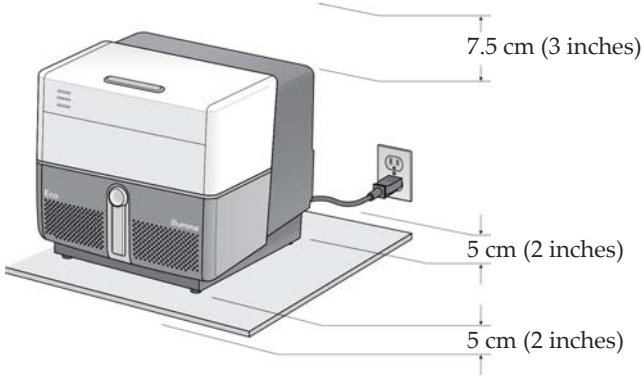
3 Check to ensure that all components are present and intact.
Your system comes with:



A Eco instrument
B Netbook computer
C Plate seals (box of 40)
D Dock and squeegee
E Plates (bag of 10)
F Ethernet cable
G USB drive
H Power cords (2)
I Mini mouse

Place Eco on the Bench

Figure 9 Eco Space Requirements

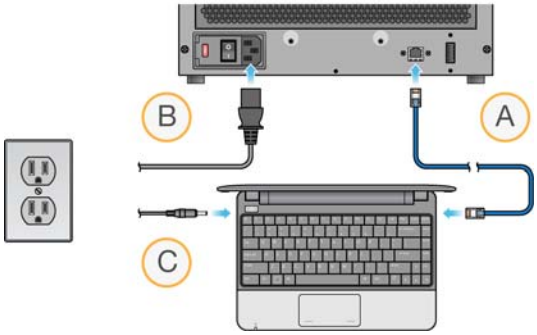


Eco requires 5 cm (2 inches) of unimpeded space at the front and back for ventilation and 7.5 cm (3 inches) above the instrument so that the lid can be opened safely.

Make sure you have easy access to the power switch on the lower right back corner of the Eco instrument and that there are two wall outlets (100-240 VAC, 50/60 Hz, 5A) within 2 m (6 feet) of the instrument.

Connect Eco

Figure 10 Eco Connections



1 Connect one end of the Ethernet cable to the Ethernet port on the netbook. Connect the other end to the Ethernet port on the rear panel of the Eco (A).

2 Connect the Eco power cord to the AC power inlet on the rear panel, and then to the wall outlet (B).

3 Connect the netbook power cord to the wall outlet (C).

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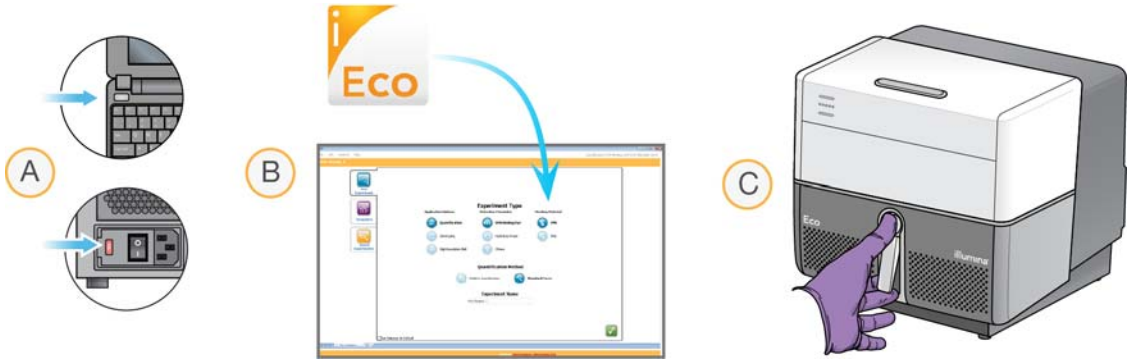
Turn on the Eco System

Figure 11 Eco Startup Sequence



1. Turn on the netbook computer, wait up to five minutes for Microsoft Windows to boot fully, then turn on the Eco instrument (A).

   The instrument runs a series of self-tests that take up to 20 minutes.

2. At any time after turning on the instrument, double-click the Eco icon on the computer desktop to start the Eco software (B).

   Communication between the netbook and the Eco instrument will be established within five minutes.

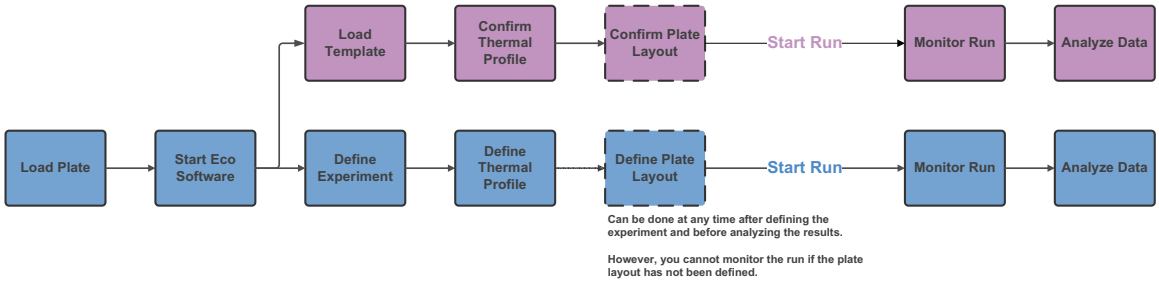
   When the READY indicator lights on the front panel stop flashing and remain solid, the instrument is ready.

3. Open the Eco by pressing the round silver button on the front to raise its handle, while lifting the handle from the bottom until the Eco pops open (C).

Workflow

Chapter 3

Eco System Workflow



1 Prepare the sample plate, load it into the Eco, and close the lid (page 15).

2 Launch the Eco software on the netbook PC.

3 Define and name the experiment by selecting the application, detection chemistry, starting material, and specific method for your application (page 16).

TIP

To use a pre-defined thermal profile and plate layout for your experiment, click **Templates** and select one of the template experiments saved on your netbook.

4 Review the thermal profile and adapt it if needed (page 17).

5 Set up the plate layout by defining assays, samples, and standards and assigning them to wells (page 18).

6 Start the run. The Monitor Run tab opens (page 26).

WARNING

Do not open the lid while a run is in progress. This allows extraneous light into the system and will corrupt the data.

7 When the run is complete, open the Eco lid. Press the plate release lever and remove the plate from the block. Dispose of any hazardous materials in biohazard, caustic material, or other appropriate containers, according to your local safety regulations.

Load the Plate

1. Thaw all necessary reagents (templates, primers, probes, and master mix).
2. Turn on the netbook PC, then the Eco, and wait until the Eco **Ready** light is solid blue.
3. Confirm that the block and optical path are clear of visible contaminants and there is no physical damage to the system, such as dents, frayed cords, or damaged levers.
4. Place a 48-well plate into the Eco sample loading dock, aligning the notch with the matching indentation on the adapter.
5. Turn on the dock light and incline the dock to a comfortable angle for pipetting.
6. Pipette samples and qPCR reagents into the plate according to your protocol.

WARNING

Wear protective gloves and eyewear when handling any material that might be considered caustic or hazardous.

7. Seal the plate with an Eco optical seal. Holding the plate in place on the Eco sample loading dock, drag the squeegee firmly across the surface to ensure the seal is secure.
8. Place the plate adapter with your loaded and sealed plate into a compatible centrifuge rotor along with the second supplied plate adapter for balance. Centrifuge the plate at 250 g for 30 seconds. Do not spin more than 500 g. Verify that there are no air bubbles at the bottom of the wells.
9. Open the Eco lid and place the plate on the block, aligning the notch against the top-left corner.

WARNING

Forcing the plate into any other orientation could damage the instrument.

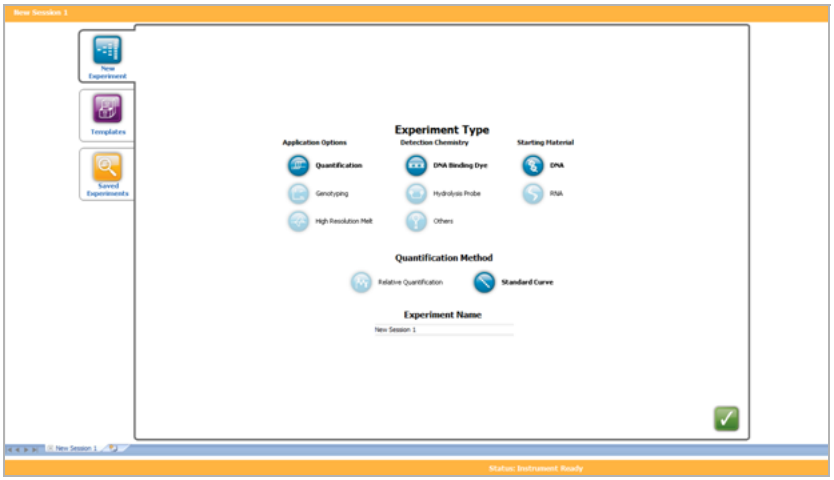
WARNING

Be careful not to touch the heated lid above the plate. It heats to 105°C (221°F) when the instrument is turned on and could result in burns.

10. Close the Eco lid. The heated lid automatically creates a seal around and on top of the plate to prevent evaporation.
11. Proceed to define a new experiment.

Define a New Experiment

Figure 12 New Experiment Tab



1 Double-click the Eco icon on the desktop to open the software. The New Experiment tab opens.

2 Select an **Application Option**, **Detection Chemistry**, and **Starting Material**. When you select the application, the software automatically configures options for downstream setup and analysis. For example, High Resolution Melt (HRM) is associated with DNA Binding dyes and so the other two detection chemistries are grayed out for High Resolution Melt experiements.

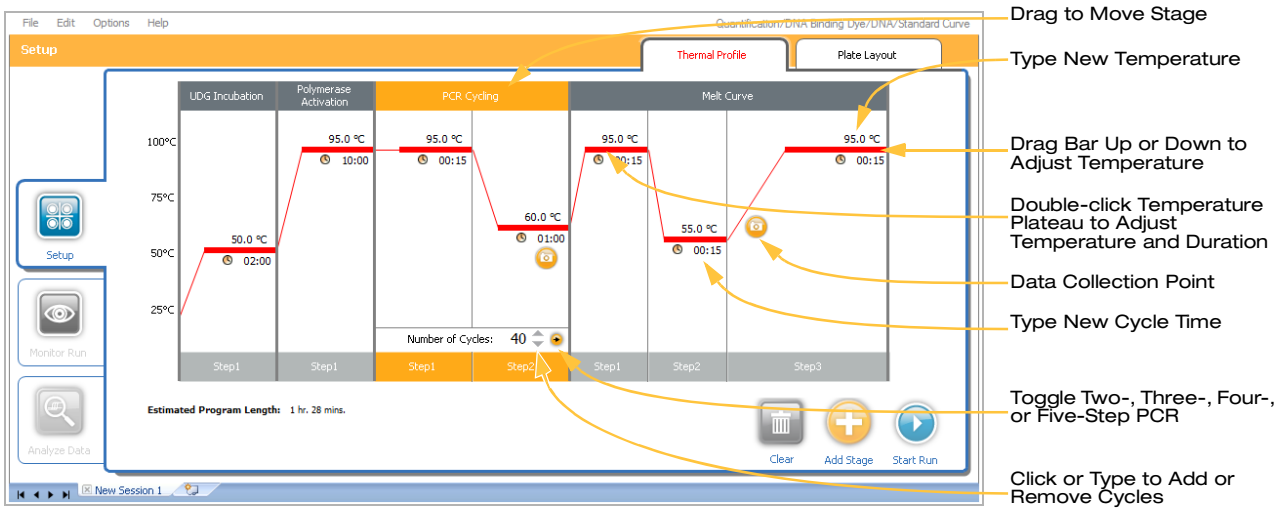
3 Select the specific method or protocol you want to use for your experiment:

Experiment Type	Options
Quantification	**Relative Quantification** or **Standard Curve**
Genotyping	**Genotyping PCR** or **Genotyping Single-Read**
High Resolution Melt	**PCR with HRM Curve** or **HRM Curve Only**

4 Enter an experiment name of up to 20 characters.

5 Click . The Setup window opens, with the Thermal Profile tab visible.

Set Up the Thermal Profile



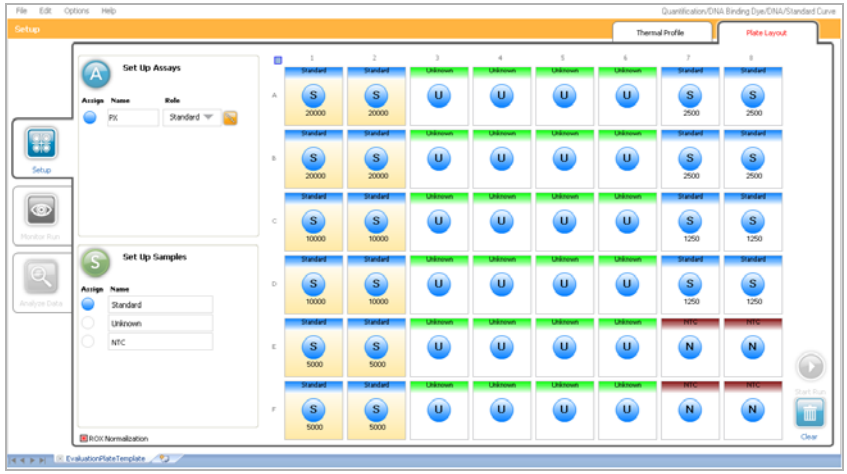
When you define the experiment a corresponding default thermal profile is selected automatically. You can use this or modify it based on your reagent's recommended protocol. You can set up cycle parameters in the Thermal Profile at any time after defining the experiment, but only before starting the run.

- Click ⊕ to add a new stage, such as a reverse transcription incubation at the beginning or additional PCR Cycling stages. The stage will appear at the end of the cycle and you can drag it to the desired location.

  Alternatively, you can drag the ⊕ icon to the location within the profile where you would like the new stage to be added.
- The camera icon indicates when the Eco collects image data. In multi-step PCR, you can select whether to collect data at the annealing or extension step. Extension is the default. To move it to annealing, mouse over the annealing step and click the dim camera icon that appears.
- To remove a stage, drag it to the trash can or highlight it and press **Delete**.

Define the Plate Layout

Figure 13 Plate Layout Tab



The Plate Layout tab lets you define how your samples, assays, and standards are laid out on the plate loaded in the Eco. The analysis software uses the plate layout to calculate data values. Plate layout involves the following steps:

1 Set up assays (page 20)

2 Set up samples (page 21)

3 Assign assays and samples to wells (page 22)

4 Define standards (Standard Curve Quanitification experiments only) (page 23)

5 Specify whether or not to do ROX Normalization

You can lay out the plate any time between defining the experiment and analyzing the data. However, you will only be able to see deconvoluted data while monitoring the run (page 25) and will not be able to analyze data (page 26) until the plate layout is defined.

Assays and Reporter Dyes

An assay is the set of primers or primers/probe used to quantify a nucleic acid target sequence. Assays can have different roles, such as Unknown, Standard, Negative, Positive, or NTC (Non-Template Controls).

Each assay is associated with a reporter dye which identifies the assay during analysis. Reporter dyes can belong to one of four "channels," each of which is defined by a specific excitation and emission range.

You can assign up to four assays per well. Within each well, assays cannot use reporter dyes from the same channel (see following table). If they did, data from assays using the same channel would be indistinguishable during analysis. A red outline around a well indicates that it contains more than one reporter dye from the same channel and requires correction before you can analyze your data.

Channel	Excitation (nm)	Emission (nm)	Fluorophores Calibrated on the Eco (Reporter)
1	452–486	505–545	SYBR Green I, FAM
2	542–582	604–644	ROX[a]
3	452–486	562–596	HEX, VIC
4	542–582	665–705	Cy5

a. If you use ROX as a passive reference for normalization, your plate layout cannot include an assay whose reporter dye is measured in channel 2.

Set Up Assays

1 On the Plate Layout tab, click **Set Up Assays** to open the Assays dialog box.

2 Select the number of assays.

3 For each assay:

- Define a name and color.
- **For Genotyping experiments**: Select **Bases** for Alleles 1 and 2.
- Select a **Reporter** dye, thereby setting the channel.

  If your dye is not listed, select a reporter with the most similar excitation and emission range to your dye (refer to the Channel table on page 6).

  For Genotyping experiments: Select a reporter dye for Alleles 1 and 2.
- Select a **Quencher**. Quencher molecules absorb fluorescent emissions of reporter dyes when in close proximity.

  By default, the quencher is set to **None** for DNA binding dye chemistry and **Non-fluorescent** for Hydrolysis probes. Note that BHQ and MGB are considered non-fluorescent quenchers.

  For Genotyping experiments: Select a quencher for Alleles 1 and 2.
- **For Relative Quantificaton experiments**: Specify the **PCR Efficiency (%)**. This can be any numeric value between 0-200%.

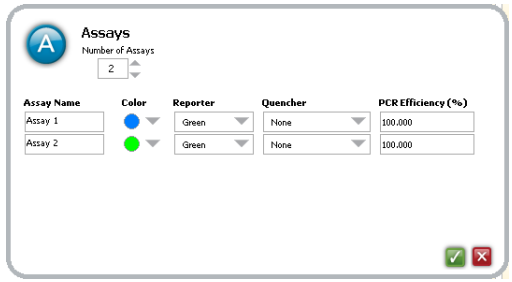
4 Click to close the Assays dialog box and return to Plate Layout.

5 **For Relative Quantification experiments**: Select at least one **Reference** assay.

6 **For Genotyping and High Resolution Melt experiments**: If you want to use controls, select the control type for each assay from the Role drop-down menu.

7 Proceed to set up samples.

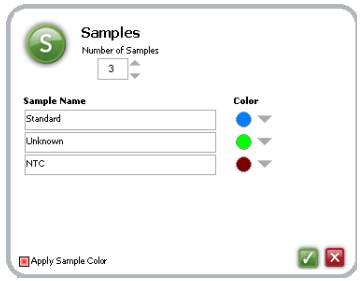
Figure 14 Assays Dialog Box, Relative Quanitfication Experiments



Set Up Samples

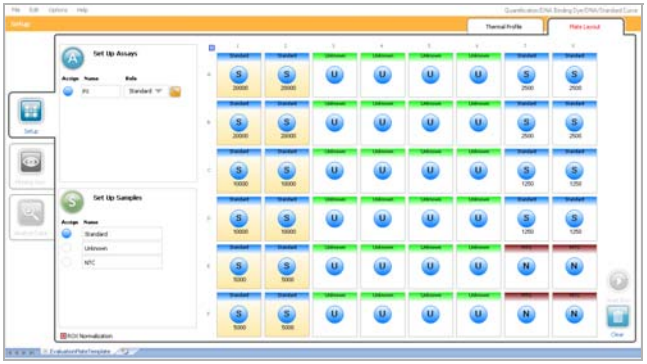
1. On the Plate Layout tab, click **Set Up Samples** to open the Samples dialog box.
2. Select the number of samples.
3. For each sample, define a name and color.
4. Click to close the Samples dialog box and return to Plate Layout.
5. **For Relative Quantification experiments**: Select at least one **Reference** sample.
6. Proceed to assign assays and samples to wells.

Figure 15 Samples Dialog Box



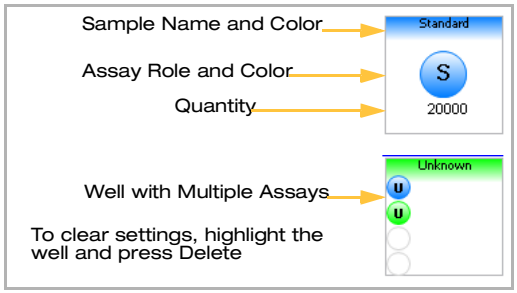
Assign Assays and Samples to Wells

Figure 16 Plate Layout Tab, Assigning Assays and Samples



1 Left-click and drag the mouse to highlight one or more wells on the plate layout diagram. Wells turn gold when they are highlighted, as shown in columns 1 and 2 of Figure 16.

2 Click the **Assign** button for up to four assays and one sample in the left pane of the window to assign the assays and sample to the highlighted wells.



3 To change the role of an assay in a given well, highlight the well and then select the desired **Assay Role** from the drop-down list.

NOTE

For quanitification experiments that will be combined using the EcoStudy software, for at least one plate in the study, you must specify:

- Standard Curve studies: At least two wells with the role “Standard”, but with different quantities
- Relative Quantification studies: At least one well with the role “Unknown” or “Positive” and a sample assigned

Any plate meeting these specifications can be used as the *mother plate* in your study. (The mother plate is the plate against which the other experiments in the study will be compared.)

4 **For Standard Curve experiments**: Proceed to define standards.

For other experiments: Click to start the run now.

Define Standards

When you set an Assay Role to Standard, a small orange Standards button appears to the right of the assay role.

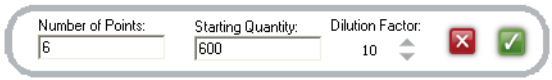
1 Click Standards to open the Set Up Standards pane in the lower left of the window.

2 Select the units that are used in your standards, and then enter the quantity.

Figure 17 Set Up Standards Pane

Auto-Calculate Serial Dilutions

1 To auto-calculate serial dilutions, click Define Standards. The Dilutions dialog box opens.



2 Enter the number of points in the standard curve, the quantity of the most concentrated standard, and the desired dilution factor, and then click .

Manually Enter Dilutions

1 Enter the value of the first standard into the first **Quantity** field below Units.

2 Press **Enter** to commit the value and open the next **Quantity** field.

Assign Standard Dilutions to Wells

You can assign standard dilutions to wells manually or automatically.

To assign dilutions automatically

1 Left-click and drag the mouse over a group of Standard Assay wells.

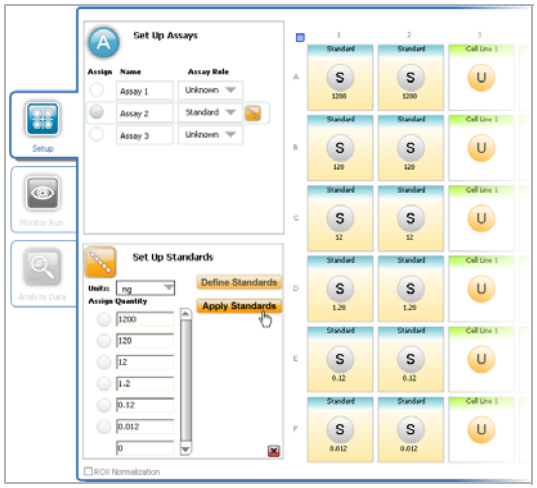
Vertical Wells = # Points on Standard Curve

Horizontal Wells = # Replicates

The **Apply Standards** button becomes active when you have selected a suitable group of wells.

2 Click **Apply Standards**. The dilutions and replicates are automatically added in the highlighted group of wells.

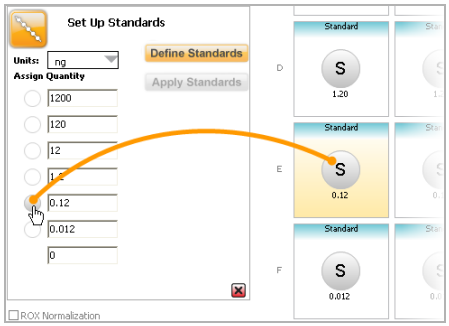
Figure 18 Selecting Standard Assay Wells



To assign dilutions manually

Highlight a Standard Assay well and click the **Assign** button beside the appropriate dilution quantity (Figure 19).

Figure 19 Assigning Dilutions



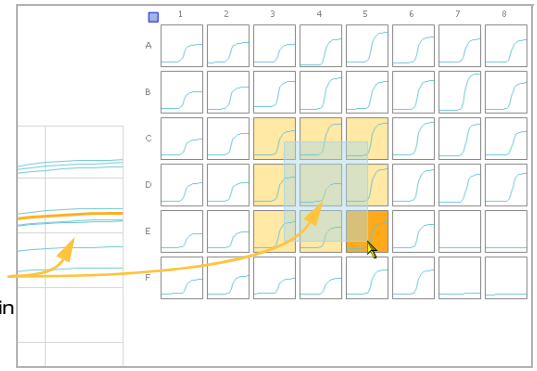
Monitor Run

WARNING

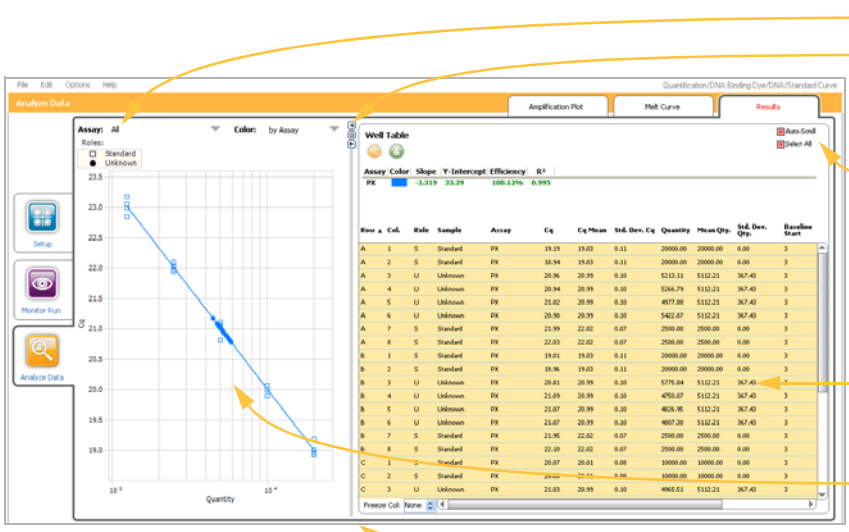
Do not open the lid while a run is in progress. This allows extraneous light into the system and will corrupt the data.

NOTE

If you have not yet defined the plate layout (page 18), you will only be able to view progress against the Thermal Profile on this tab.



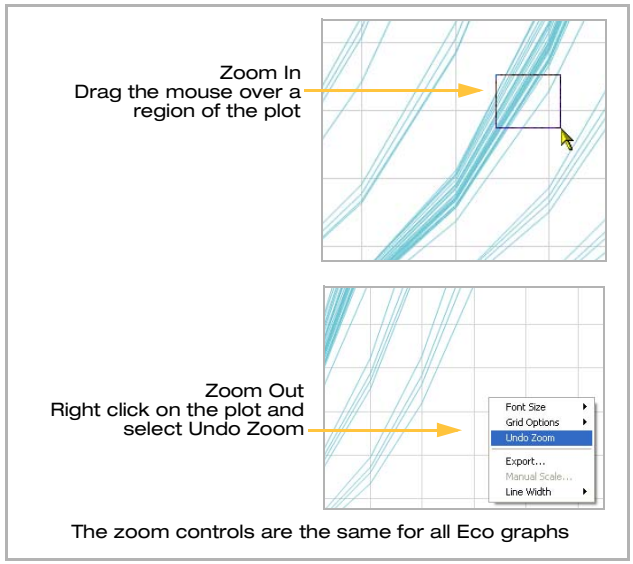
Analyze Data



There are three tabs:

- Amplification Plot
- Melt Curve
- Results

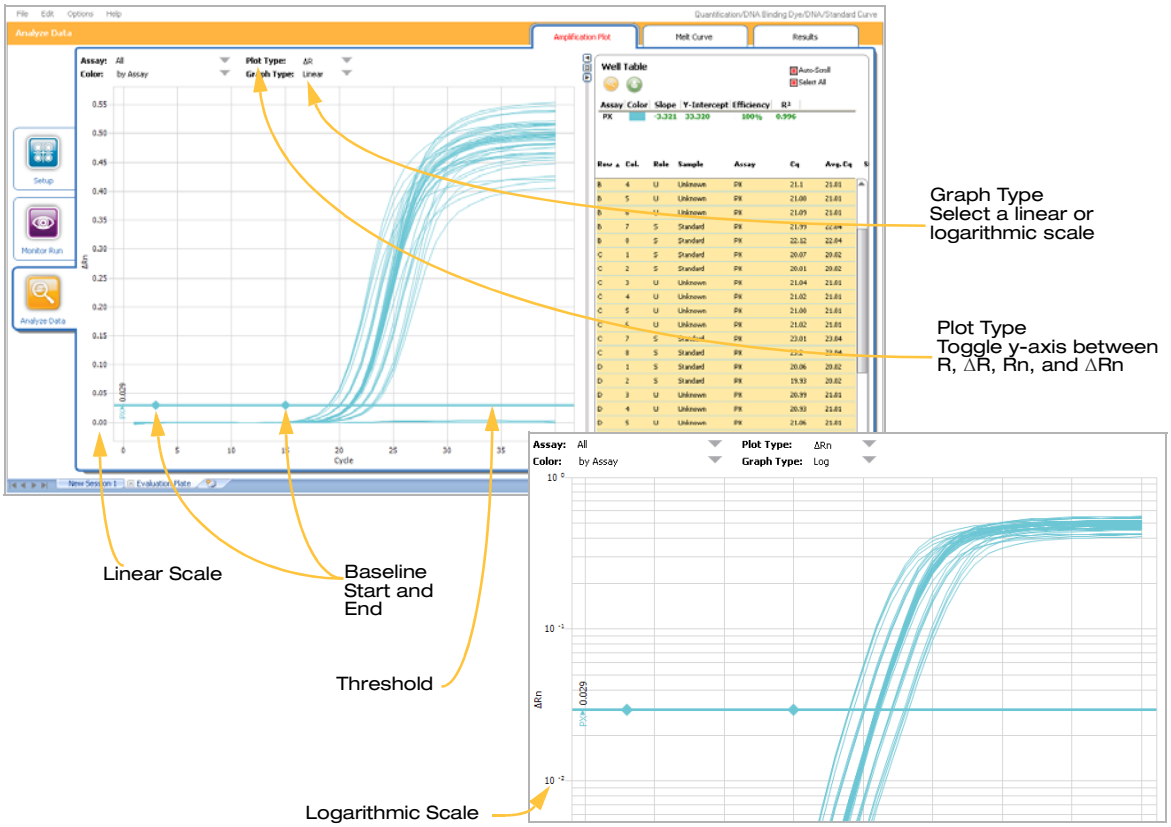
The window controls are the same for each tab.



Amplification Plot Tab

The Amplification Plot tab displays RFU versus the cycle number. You can toggle betwen showing deconvoluted RFU (**R**) or baseline-subtracted R (**ΔR**), or, if ROX is used, you can toggle between showing R normalized to the ROX fluorescence level (**Rn**) or baseline-subtracted Rn (**ΔRn**).

Figure 20 Amplification Plot Tab



Baseline Correction

In a qPCR reaction, DNA is amplified and tracked using a fluorescent reporter dye. The point at which the amplification signal crosses a threshold is used to estimate the amount of DNA present at the start of the reaction. Typically, the signal measured in a qPCR reaction has two components. The first is due to the DNA synthesis, and the second is a background component that grows linearly with PCR cycle. To analyze qPCR data appropriately, the linear component must be identified and subtracted from the signal.

The Eco software uses a modification of the method of Liu and Saint (2002) to model the fluorescence plot as a line and a sigmoid. The model-fitting algorithm finds and subtracts the linear portion of the curve, and then the sigmoidal component is estimated.

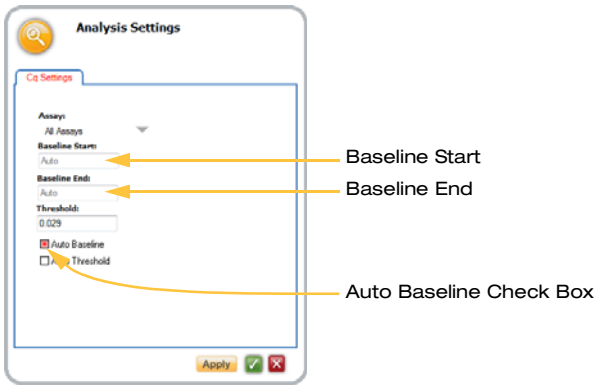
NOTE

The auto-baselining algorithm needs at least ten cycles of data in order to perform baseline subtraction using this method. Reactions with fewer than ten cycles of data are treated with a different auto-baselining algorithm (not described here).

You can manually adjust the baseline parameters on a per assay basis across all wells to optimize the quality of the data, either numerically from within the Analysis Settings dialog box or graphically.

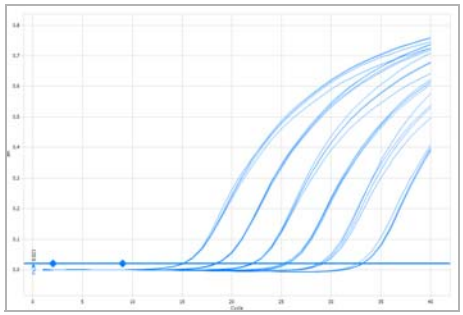
- To select the method of baseline determination, click **Analysis Settings** in the right panel or select **Options** | **Analysis Settings** from the main menu bar. The Analysis Settings dialog box opens. Click the Auto Baseline check box to toggle between auto and manual baseline determination.

Figure 21 Analysis Settings Dialog Box



- To adjust baseline numerically, enter the desired **Baseline Start** and **Baseline End** cycles in the Analysis Settings window, then click Apply .
- When auto baseline is deactivated, the baseline start and stop cycles are indicated graphically by the appearance of filled diamonds along the threshold line for each assay. To adjust baseline graphically, drag ◆ diamonds horizontally to indicate the interval boundaries. Typically, the stop cycle should be placed 2 cycles prior to the start of the exponential phase of the amplification.

Figure 22 Baseline Adjustment



Threshold Adjustment

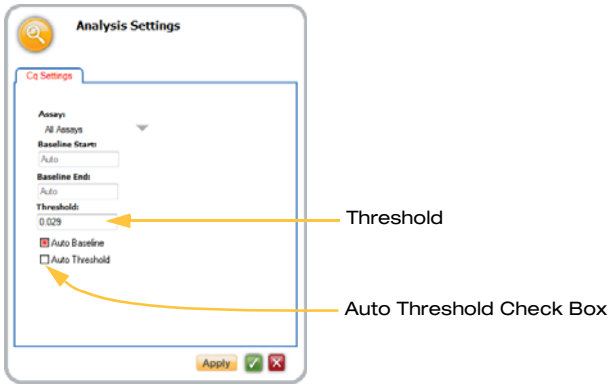
The Eco software uses a modification of the method of Liu and Saint (2002) to model the fluorescence plot as a line and a sigmoid. The model-fitting algorithm finds and subtracts the linear portion of the curve, and then the sigmoidal component is estimated.

The auto-threshold algorithm determines the threshold value by finding a threshold such that most reactions are at roughly the same point in the sigmoid portion of the model, which corresponds to roughly the same point in the progress of the reaction toward saturation.

This threshold can be adjusted manually to optimize the quality of the data, either numerically from within the Analysis Settings dialog box or graphically.

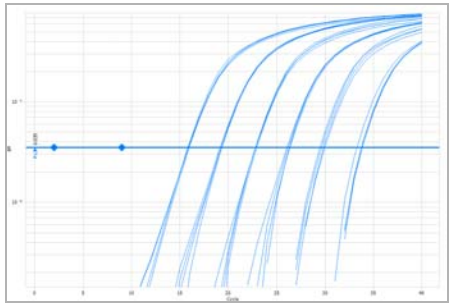
- To specify whether the threshold is determined automatically or manually, open the Analysis Settings dialog box and click the **Auto Threshold** check box to toggle between auto and manual threshold determination.

Figure 23 Analysis Settings Dialog Box



- To adjust threshold numerically, enter the desired **Threshold** value in the Analysis Settings dialog box, then click Apply .
- To adjust threshold graphically, drag the horizontal bar up or down into the exponential growth phase of the curve.
  - In a linear scale view, this needs to be set close to the inflexion point of the amplification plots.
  - In a log scale view, it should be set in the middle of the exponential phase, as shown in Figure 24.

Figure 24 Threshold, Log Scale View



Melt Curve Tab

This tab is active if you ran a melting curve in your thermal profile while using DNA binding dyes such as SYBR green.

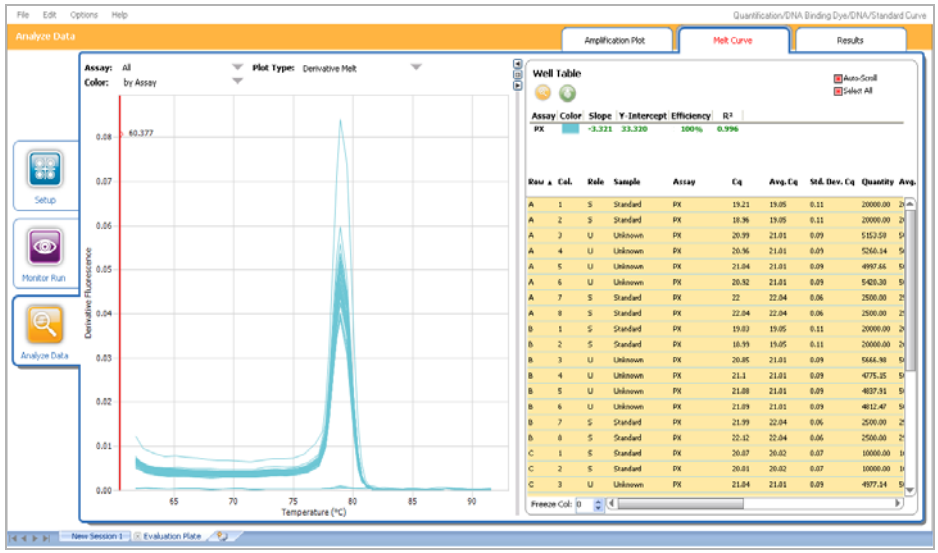
The Melt Curve tab displays the negative derivative of RFU versus temperature (-dRFU/dT) and calculates melting temperatures (Tm) based upon peak calls.

Tm calls (up to three per well) are listed in the Well Table on the right side of the tab and are ranked based upon maximum amplitude. This is useful for calling out primer dimers and mispriming, especially if only one amplicon is expected.Visualizing melt peaks can be aided by dragging the red vertical bar horizontally and zooming in on desired areas within the graph.

Standard Melt

For quantification experiments, a standard melt is performed that collects fluorescence data every 0.3°C during melting. You can toggle the graph between showing the raw and derivative views of melt curves.

Figure 25 Melt Curve Tab, Standard Melt

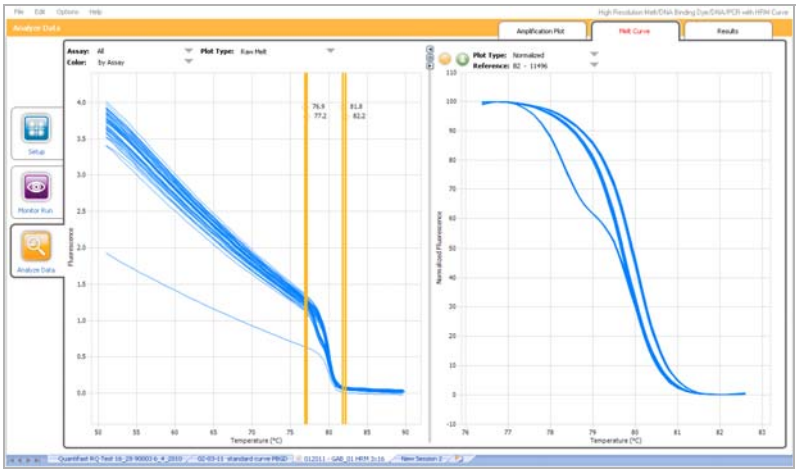


High Resolution Melt

For HRM experiments, fluorescence data is collected every 0.1°C and is presented in two distinct graphic representations. The identity of the well associated with each curve is displayed at the top of each graph when mousing over an individual curve.

Pre- and Post-Melt normalization regions are set to define the temperature boundaries of the normalized and difference plots. The average value of the pre-melt region is used as the 100% signal in the normalized view; the average value of the post-melt region is used as the 0% signal. These regions can be defined within the graph by using the mouse to drag the orange vertical bars horizontally to flank the temperature range where product melting occurs. Pre and post-melt normalization regions can also be defined in the the Analysis Settings dialog box.

Figure 26 Melt Curve Tab, High Resolution Melt



By default, the left panel displays the raw melt data, but it can be toggled between raw and derivative views of melt curves.

By default, the right panel presents the normalized view of HRM data, but it can be toggled between the Normalized View and the Difference Plot using the Plot Type drop-down menu. The plot type selected for the right panel of the melt tab determines the default view when navigating to the Results tab.

To view a Difference Plot, you must specify a reference well. The reference well can be set using the Reference drop-down menu within the graph, the HRM settings tab of the Analysis Settings dialog box, or the right-click menu when mousing over a well on the Plate Layout tab.

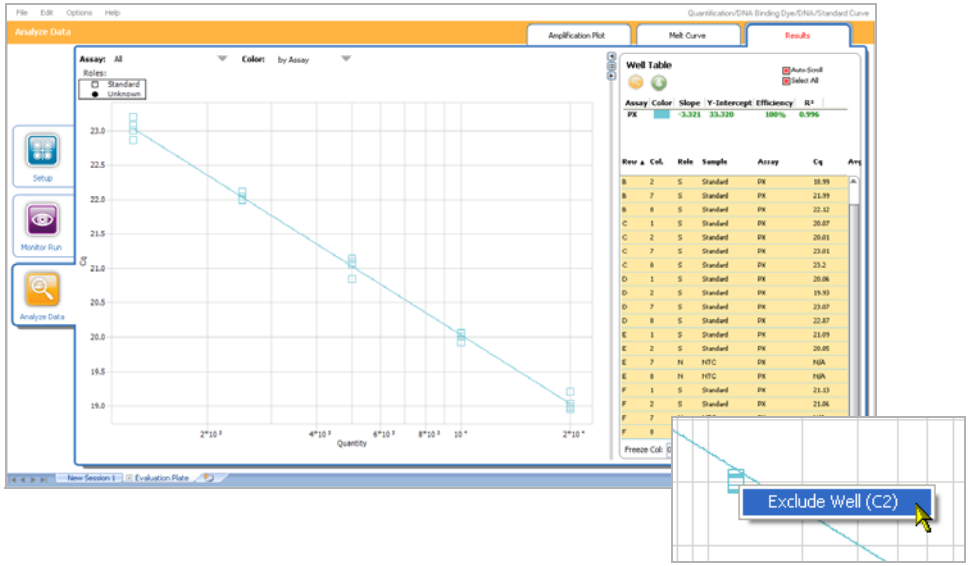
Results Tab

Eco software automatically analyzes the data and generates a plot based upon the experiment type along with any baseline or threshold adjustments.

Standard Curve

Standard Curve experiments generate a standard curve (Figure 27). The slope, PCR efficiency, and R^2 of that curve appear in a table above the Well Table in the right panel. In some cases you might want to exclude outlier data from the analysis. Right-click the data point in the graph or the well location in the table and select **Exclude Well** from the context menu (see inset). The Well Table will list the well as **Excluded**.

Figure 27 Results Tab, Example of a Standard Curve display



Relative Quantification

Relative quantification experiments generate a bar graph that includes error bars. The displayed histogram view can be toggled to group data by assay or by sample. Expression levels are normalized by user-indicated reference assays and are plotted relative to the reference sample. Reference assays and sample can be set within the Plate Layout tab or within the Analysis Settings dialog box.

Moving the cursor over one of the bars on the histogram reveals a text box displaying the relative expression value for that bar. By default, PCR efficiencies of each assay are set to 100%. Previously determined values for PCR efficiencies can be entered as percentages on the RQ Settings tab in the Analysis Settings dialog box.

In scenarios where both singleplex and multiplex wells are included on a single Eco plate, you must select whether to perform the data analysis using either the singleplex or multiplex methodology.

Figure 28 Results Tab, Example of a Relative Quanitification display

Relative Quantification Calculations

The Eco Real-Time PCR System supports relative quantification using various mathematical models. All methods rely on normalization against a reference gene or a panel of multiple reference genes as well as a reference or control sample.

Normalization to a Single Reference Gene

The default method is the ΔΔCq method, also known as the Livak method (Livak, et al., 2001). This method normalizes the expression of the target genes relative to a single reference gene and expressed relative to a reference sample. The exact calculations are adapted from Livak, et al. and are summarized below.

$$\Delta Cq = AVE\ Cq_{(Target\ Assay)} - AVE\ Cq_{(Reference\ Assay)}$$

$$\Delta\Delta Cq = \Delta Cq_{(Test\ Sample)} - \Delta Cq_{(Reference\ Sample)}$$

$$RQ = 2^{-\Delta\Delta Cq}$$

This method assumes that the amplification efficiencies of the target and reference genes are equal and at 100%. Before using the ΔΔCq method, it is important to experimentally validate these assumptions by determining the amplification efficiencies of the target and reference genes.

If the amplification efficiencies of the target and reference genes are not equal, an alternative method is used. The Pfaffl method (Pfaffl, 2001), does not assume equal or 100% amplification efficiency and incorporates the experimentally determined efficiencies of the target and reference genes to correct for any differences. The calculations for this method are shown below.

$$\Delta Cq = AVE\ Cq_{(Ref\ Sample)} - AVE\ Cq_{(Unk\ Samples)}$$

$$Quantity = (Efficiency)^{\Delta Cq}$$

$$RQ = Quantity_{(Target\ Assay)} \div Quantity_{(Reference\ Assay)}$$

(Efficiency) is the experimentally determined amplification efficiency of the target or reference gene. (The Pfaffl and Livak methods are related. The Livak method is essentially the Pfaffl method where $E_{(target)} = E_{(ref)} = 2$.)

Normalization to Multiple Reference Genes

In addition to normalizing to a single reference gene, the Eco system supports normalization to multiple reference genes (Vandesompele, et al., 2002). This method uses the geometric mean of a reference gene panel to determine a normalization factor. The calculations for this method are shown below.

$$\Delta Cq = AVE\ Cq_{(Ref\ Sample)} - AVE\ Cq_{(Unk\ Samples)}$$

$$Quantity = (Efficiency)^{\Delta Cq}$$

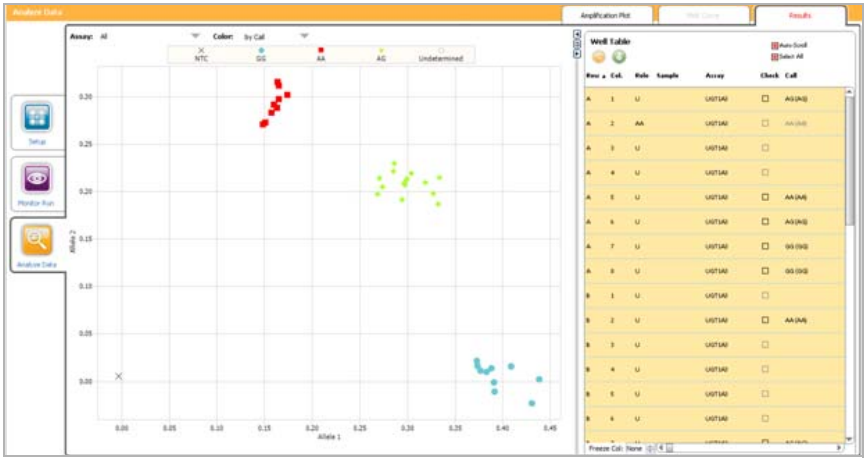
$$Normalization\ Factor = Geometric\ Mean_{(QuantityRef\ Assay1,\ QuantityRef\ Assay2,...)}$$

$$RQ = Quantity\ Target \div Normalization\ Factor$$

Allelic Discrimination

Allelic discrimination (Genotyping) experiments generate a scatter plot (Figure 29). Bi-allelic genotyping results in three potential clusters of end-point fluorescence data. Allele names are user-defined within the Control Types dialog box, available from the **Options** menu at the top of the screen.

Figure 29 Results Tab, Example of a Allelic Discrimination display



When controls are used, autocalling of clusters can be toggled on and off within the Genotyping Settings tab of the Analysis Settings dialog box. When autocalling is active, the Confidence Value of genotyping calls can be modified within the text field below the autocalling selection.

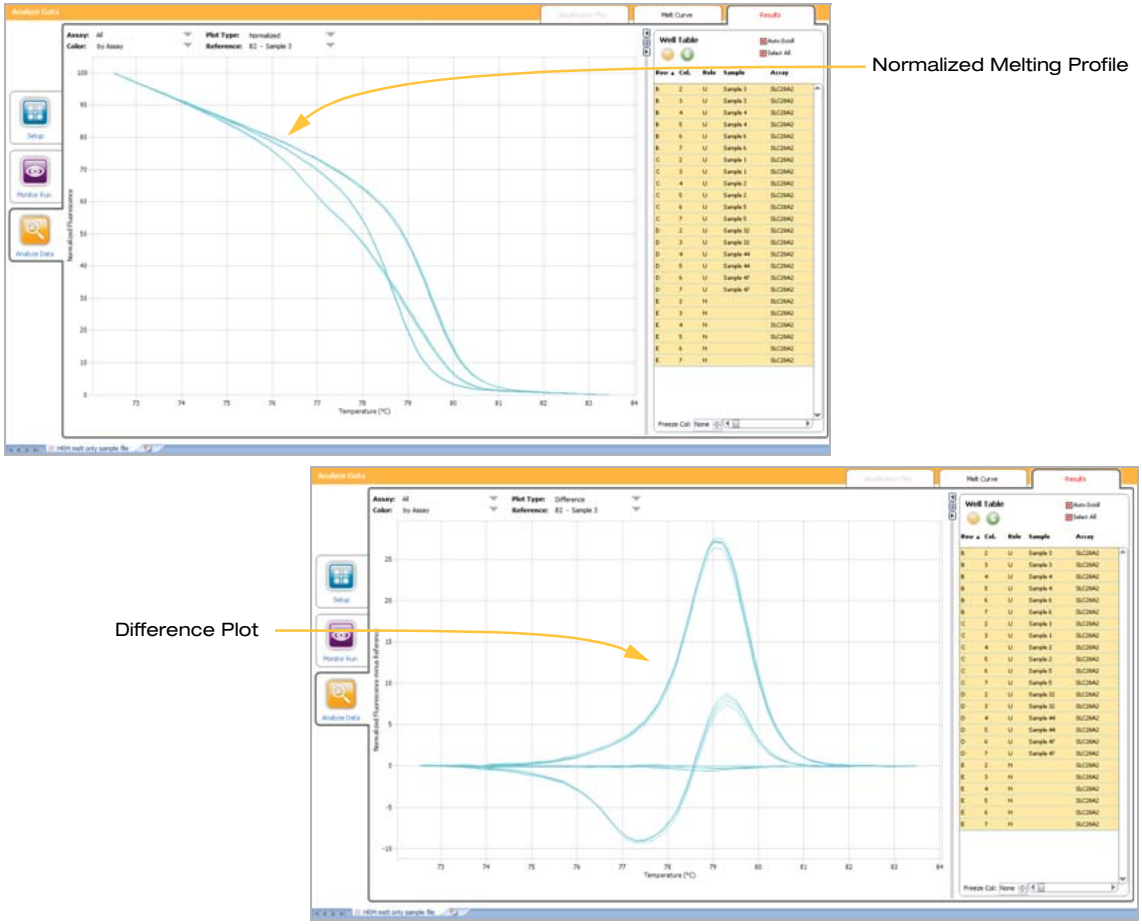
NOTE

To ensure accurate genotype calling, it is highly recommended that you include No Template Controls (NTCs) in your experiment.

High Resolution Melt

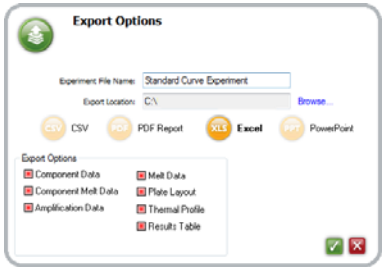
HRM experiments generate a melting curve graph, which can be displayed as either a normalized melting profile or a difference plot (Figure 30) to maximize cluster resolution. The Difference Plot displays melt curves relative to the sample in the well selected from the Reference dropdown menu at the top of the graph.

Figure 30 Results Tab, Example of High Resolution Melt displays



You can manually assign a call to a given sample by holding down the Shift key, left-clicking on the graph, and drawing a line across the melt curve. Then, either in the Well Table or by right-clicking on the graph, select the appropriate genotype.

Export Results and Data



Results, as well as other data, can be exported by clicking above the Well Table or by selecting **File** | **Export** in the main menu.

In the Export Options dialog box (shown left), select the desired file format and components to export, then click .

System Information

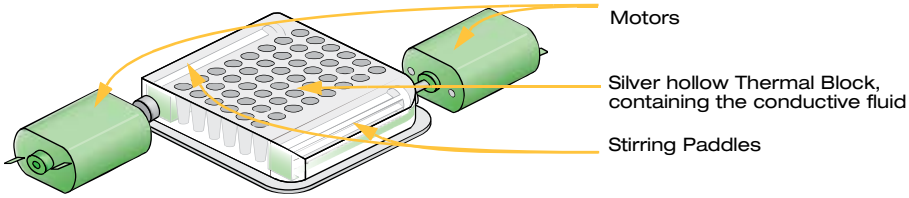
Chapter 4

Components

Thermal System

- Proprietary hollow silver thermal block filled with circulating conductive fluid provides superior temperature control and thermal uniformity across the sample plate
- Standard Fast protocol performs 40 PCR cycles in approximately 40 minutes

Figure 31 Eco Thermal System

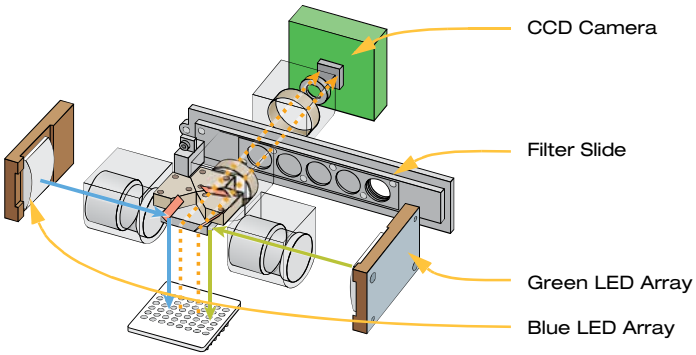


Optical System

- Two LED arrays provide individual sample well excitation
- Four detection filters support almost all PCR chemistries and multiplex detection (ROX is optional)
- CCD camera acquires high-quality data in all wells and filters at each PCR cycle

Factory-calibrated optics support SYBR Green, FAM, HEX, VIC, ROX, and Cy5 dyes. You can also use other dyes that are compatible with the excitation and emission wavelengths.

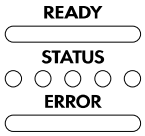
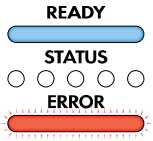
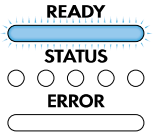
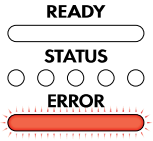
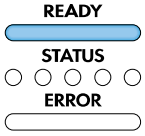
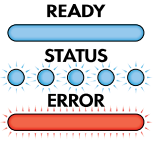
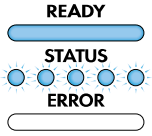
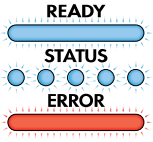
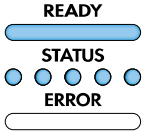
Figure 32 Optical System



Lights

The Eco System has three indicator lights on the front: **Ready**, **Status**, and **Error**. The following table shows the meaning of each combination of off, on, and flashing lights.

NOTE
Flashing lights are indicated in the table by dashed lines around the outside of the light.

Lights	Description	Lights	Description
	Power Off		Non-Fatal Error Decide whether you want to terminate the run
	Initializing (conducting self tests and heating the thermal block)		Fatal Error: Run Terminated Instrument might have overheated or encountered a hardware failure
	Ready/Idle		Software Updating
	Run In Progress **Do not switch off or open the lid while a run is in progress**		Communicating with Netbook
	Run Complete		

Specifications and Environmental Requirements

Optical	Light Source	Two sets of 48 LEDs (452-486 nm and 542-582 nm)
	Detector	CCD camera (4 filters) (505-545 nm, 562-596 nm, 604-644 nm, and 665-705 nm)
Thermal	Thermal Cycling	Proprietary hollow silver block with Peltier-based system
	Thermal Uniformity	± 0.1°C
Operational	Sample Format	48-well plate
	Reaction Volume	5–20 µl
	Warmup Time	~ 20 minutes
	Typical PCR Run Time	Less than 40 minutes for 40 cycles
	Sensitivity of Detection	1 copy
	High Resolution Melt	Supported resolution to 0.1°C
	Multiplexing	Detection of up to four targets simultaneously (four-plex)
	Passive Reference	Optional (ROX)
Physical	Dimensions	34.5 cm W x 31 cm D x 32 cm H (13.6 in. W x 12.2 in. D x 12.6 in. H)
	Weight	13.6 kg (30 lb) including power supply
Environmental	Electrical	100–240 VAC, 50/60 Hz, 5A
	Temperature Range	Operating: 15°C to 30°C (59° F to 86° F) Storage: 10°C to 100°C (50° F to 212° F)
	Humidity Range	Operating: 15–90% Relative Humidity Storage: 5–95% Relative Humidity

Symbols

	CAUTION: Hot Surface
	Do Not Throw in Trash: At end of useful life, recycle the system or device
EC REP	European Representative
	Fuse: replacement fuses must meet the stated rating
	Humidity Range (on packaging: indicates acceptable shipping and storage limits)
	Manufactured By
CE	Mark of European Conformity: device complies with the EMC Directive (2004/108/EC) and the Low Voltage Directive (2006/95/EC)
REF	Model Number
	Off
	On
SN	Serial Number
	Temperature Range (on packaging: indicates acceptable shipping and storage limits)

Electromagnetic Compatibility

This equipment complies with the emission and immunity requirements described in IEC 61326-1:2005 and IEC 61326-2-6:2005. To confirm proper operation:

- The electromagnetic environment should be evaluated prior to operation of the system.
- Do not use this system in close proximity to sources of strong electromagnetic radiation (e.g. unshielded intentional RF sources), as these may interfere with proper operation.
- If you notice any interference, discontinue using the system until all issues are resolved. Resolution may include moving cords from other equipment away from the system, plugging the system into an outlet on a different circuit from other equipment, or moving the system away from the other equipment. If you continue to have difficulties, contact Illumina.

Cleaning And Maintenance

Clean the block and housing as needed, following these directions.

CAUTION

If hazardous or biohazardous material is spilled onto or into the equipment, clean it immediately.

1 Turn the system off and allow the block to cool completely.

2 Using a lint-free cloth slightly dampened with clean water, gently wipe the surfaces of the equipment. If a stronger cleaning agent is needed, use a lint-free cloth slightly dampened with 95% isopropyl alcohol.

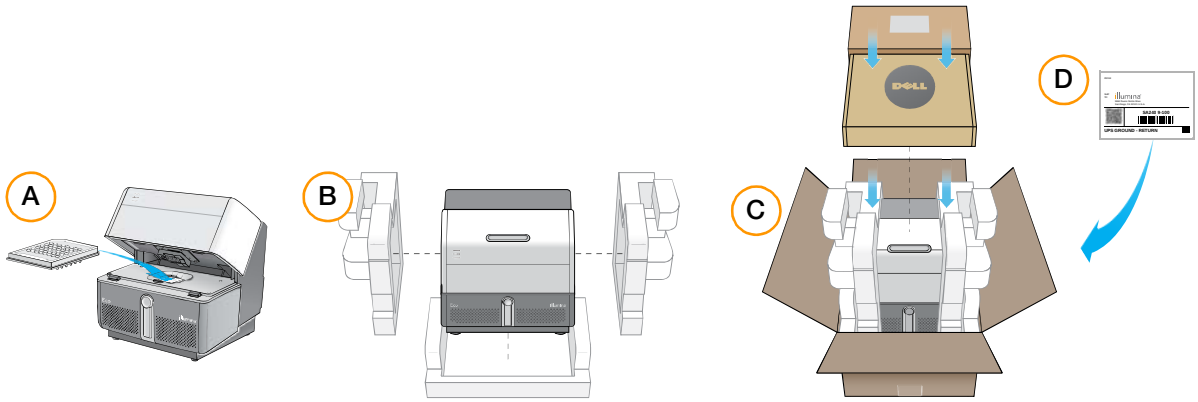
Follow these practices for proper maintenance of your Eco system.

- Every time you use the system, visually check it to confirm there is no obvious physical damage such as dents, frayed cords, or damaged levers. If you see any damage, discontinue use and contact Illumina Technical Support.
- Once a year, run a known test sample to confirm accurate analysis.

CAUTION

The Eco system contains materials that may be hazardous to the environment if not disposed of properly. Be sure to dispose of materials according to all local, state/provincial, and national regulations.

Return Process



Follow these directions if you need to return the Eco instrument to Illumina for any reason (repairs, for example.)

1. Follow the instructions in *Cleaning And Maintenance* on page 45, or provided by your technical support representative, to clean the instrument.
2. Obtain the original shipping box and packaging materials used to ship Eco to you. If you do not have them, go to **http://www.illumina.com/ecoqpcr** for instructions on ordering return shipping materials.
3. Put an empty plate into the instrument for safe shipping, and then close the lid (A).
4. Place the instrument into the white foam packaging, ensuring that it is properly positioned for complete protection in shipping (B).
5. Put the packaged instrument into the shipping box (C).
6. If requested, package the Dell netbook, dock, and squeegee into their original boxes and place the boxes on top of the Eco in the shipping box (C). You do not need to return the Evaluation Plate, buffers, extra plates, seals, or USB drive.
7. Tape the box securely for shipment and attach a shipping return label (D).

Concepts

The weight of one genome (g) = (size of genome in bp) x (618 g/mol/bp) x Avogadro's number

One human genome (g) = (3×10^9 bp) x (618 g/mol/bp) x (6.02×10^{23}) = 3.08×10^{12} g/mol

One haploid cell (sperm/egg) = 3.08 pg of DNA

One diploid cell = 6.16 pg of DNA

There is approximately one copy of every non-repeated sequence per 3.08 pg of human DNA.

The average cell contains 10–20 pg of total RNA.

About 90–95% of total RNA is rRNA (18S, 5.8S and 28S). 1–3% is mRNA.

RNA concentration (µg/µl) = (A_{260} * 40 * D)/1000, where D = dilution factor and A_{260} = absorbence at 260 nm.

DNA concentration (µg/µl) = (A_{260} * 50 * D)/1000, where D = dilution factor and A_{260} = absorbence at 260 nm

The exponential amplification of PCR (Xn) is described by the following equation:

$$Xn = Xo * (1+E_x)^n$$

where X_n = number of target molecules at cycle n; X_o = initial number of target molecules; E_x = efficiency of target amplification; and n = number of cycles

Amplification efficiency (E_x) is described by the following equation:

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

The acceptable range of assay efficiency = 90% to 110%, or a slope between -3.1 and -3.6

A slope of -3.32 indicates 100% efficiency, meaning that the number of template molecules doubled in each PCR cycle.

Common reference genes:

- High expression: 18S ribosomal RNA (18S), Beta actin (ACTB), Beta-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphoglycerokinase (PGK)
- Medium expression: Transferrin receptor (TfR)
- Low expression: Transcription factor IID TATA binding protein (TBP) and glucuronidase (GUS)

Always validate your reference genes to ensure that the genes you are choose are stable in your experiments.

Glossary

Absolute Quantification—An assay that quantifies unknown samples by interpolating their quantities from a standard curve based on a serial dilution of a sample containing known concentration.

Allelic Discrimination—An assay that discriminates between two alleles (gene variants).

Amplicon—A fragment of DNA synthesized by a pair of primers during PCR.

Assay—The set of primers or primers/probe used to quantify an amplicon.

Baseline—The initial PCR cycles when little fluorescence signal is generated. This will be used to subtract the background.

Channel—The combination of excitation and emission spectra used to monitor amplification for a given assay.

Ct—Threshold Cycle. See Cq.

Cq—Quantification Cycle. The cycle number at which the fluorescent signal crosses the threshold. It is inversely correlated to the logarithm of the initial copy number.

Dark Quencher—A quencher without any native fluorescence. Black Hole Quencher (BHQ) dyes are an example.

Delta Rn (ΔRn)—The normalized Fluorescence of an amplification plot with background and ROX normalization dye correction.

Derivate Melt Curve—A plot of temperature (x axis) versus the derivate of fluorescence with respect to temperature (-dF/dT) (y axis). Used to analyze the Tm of an amplicon.

DNA Binding Dye—A dye that increases its fluorescence in the presence of double-stranded DNA.

dsDNA—Double-stranded DNA.

Dual-Labeled Hydrolysis Probe—See hydrolysis probe.

Dynamic Range—The range of template concentration over which accurate Cq values can be determined. Extrapolation is not recommended.

Efficiency—See Slope.

Endogenous Control—An RNA or DNA template that is naturally present in each sample.

End-Point Analysis—Qualitative analysis of PCR data at the end of PCR. Allelic discrimination assays (genotyping) are an example.

Exogenous Control—A RNA or DNA template that is spiked into each sample at a known concentration.

FAM (6-carboxy fluorescein)—The most commonly used reporter dye at the 5′ end of a hydrolysis probe.

Filter—Components used to limit the bandwidth or the excitation or emission energy to the next component of the optical path.

Fluorophore—The functional group of a molecule that absorbs energy at a specific wavelength and emits it back at a different wavelength.

Fluorescence—The immediate release of energy (a photon of light) as a result of an increase in the electronic state of a photon-containing molecule.

HEX—Carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein.

High Resolution Melt (HRM)—An enhancement of the traditional melt curve analysis which increases the detail and information captured.

Hybridization Probe—A probe that is not hydrolyzed by Taq polymerase. Hybridization probes can be used for melt curve analysis. Examples include Roche FRET and Molecular Beacons.

Hydrolysis Probe—A probe that is hydrolyzed by the 5′ endonuclease activity of Taq polymerase.

Internal Positive Control (IPC)—An exogenous control added to a multiplex qPCR assay to monitor the presence of inhibitors in the template.

JOE—Carboxy-4′,5′-dichloro-2′,7′ dimethoxyfluorescein.

LED—Light Emitting Diode. A light that is illuminated by the movement of electrons in a semiconductor material. LED lights do not have filaments that burn out and do not get very hot.

Linear View—A view of an amplification plot using linear dRn values (y-axis) versus PCR cycles (x-axis).

Log view—A view of an amplification plot using log dRn values (y-axis) versus PCR cycles (x-axis).

LUX Primer Set—A self-quenched fluorogenic primer and a corresponding unlabeled primer. When the primer is incorporated into DNA during PCR the fluorophore is de-quenched, leading to an increase in fluorescent signal.

Melt Curve—See Derivative Melt Curve.

Minor Groove Binders (MGBs)—dsDNA-binding agents typically attached to the 3′ end of hydrolysis probes. MGBs increase the Tm value of probes, thus leading to smaller probes.

Molecular Beacons—Hairpin probes containing a sequence-specific loop region flanked by two inverted repeats. A quencher dye at one end of the molecule quenches the reported dye at the other end. Sequence-specific binding leads to hairpin unraveling and fluorescent signal generation.

Multiplexing—Simultaneous analysis of more than one template in the same reaction.

No Template Control (NTC)—An assay with all necessary components except the template.

Normalization—The use of control genes with a constant expression level to normalize the expression of other genes in templates of variable concentration and quality.

Passive Reference—A fluorescence dye such as ROX that the software uses as an internal reference to normalize the reporter signal during data analysis.

Peltier—Element used for heating and cooling in a qPCR machine.

Quencher—Molecule that absorbs fluorescence emission of a reporter dye when in close proximity. BHQ is a quencher.

R^2 (Coefficient of Correlation)—The coefficient of correlation between measured Cq values and the DNA concentrations. It is a measure of how closely the plotted data points fit the standard curve. The closer to 1 the value, the better the fit. R^2 is ideally > 0.99.

Reference—A passive dye or active signal used to normalize experimental results.

Reference Genes—Genes with a wide and constant level of expression. Typically used to normalize the expression of other genes. Examples of commonly used reference genes: 16S/18S, GAPDH, and b-actin.

Relative Quantification—An assay used to measure the expression of a target gene in one sample relative to another sample and normalized to a reference gene.

Reporter Dye—Fluorescent dye used to monitor amplicon accumulation. This can be a dsDNA binding dye or a dye attached to a probe. Each dye is associated with a certain channel.

Rn (Normalized Reporter Signal)—Reporter fluorescent signal divided by fluorescence of the passive reference dye.

ROX (carboxy-X-rhodamine)—The most commonly used passive reference dye.

Slope—The slope of a standard curve. It is a measure of assay efficiency. $E = 10^{(-1/slope)}-1$, where a slope of -3.32 is equal to 100% efficiency (E) or an exact doubling of template molecules in each PCR cycle. Acceptable efficiencies range from -3.6 (90%) to -3.1 (110%). Overly high efficiencies indicate qPCR inhibition, usually due to contaminants in the sample. Overly low efficiencies typically indicate problems with the reaction mix concentration.

Standard—A serial dilution of a target of known concentration used as template to generate a standard curve.

Standard Curve—A plot of Cq values against the log of target amount. Used to determine an assay's dynamic range, efficiency (slope), R^2, and sensitivity (y-intercept).

Standard Deviation (SD)—The SD of replicate Cq measurements is a measure of the precision of the assay.

Target—The DNA or RNA sequence to be amplified.

Template—See Target. Template can also refer to a saved experiment that can be used as a model for new experiments in the software.

Threshold—A level set above the background signal generated during the early cycles of qPCR. When adjusted manually, it should be set in the middle of the exponential stage of qPCR.

TET—Carboxy-2',4,7,7'-tetrachlorofluorescein.

Tm—The temperature at which 50% of dsDNA is single-stranded (melted).

Unknown—A sample containing an unknown amount of template.

Y-Intercept—In a standard curve, the value that crosses the y-axis at x = 1 (single copy target).

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Technical Assistance

For technical assistance, go to **http://www.illumina.com/ecoqpcr**.

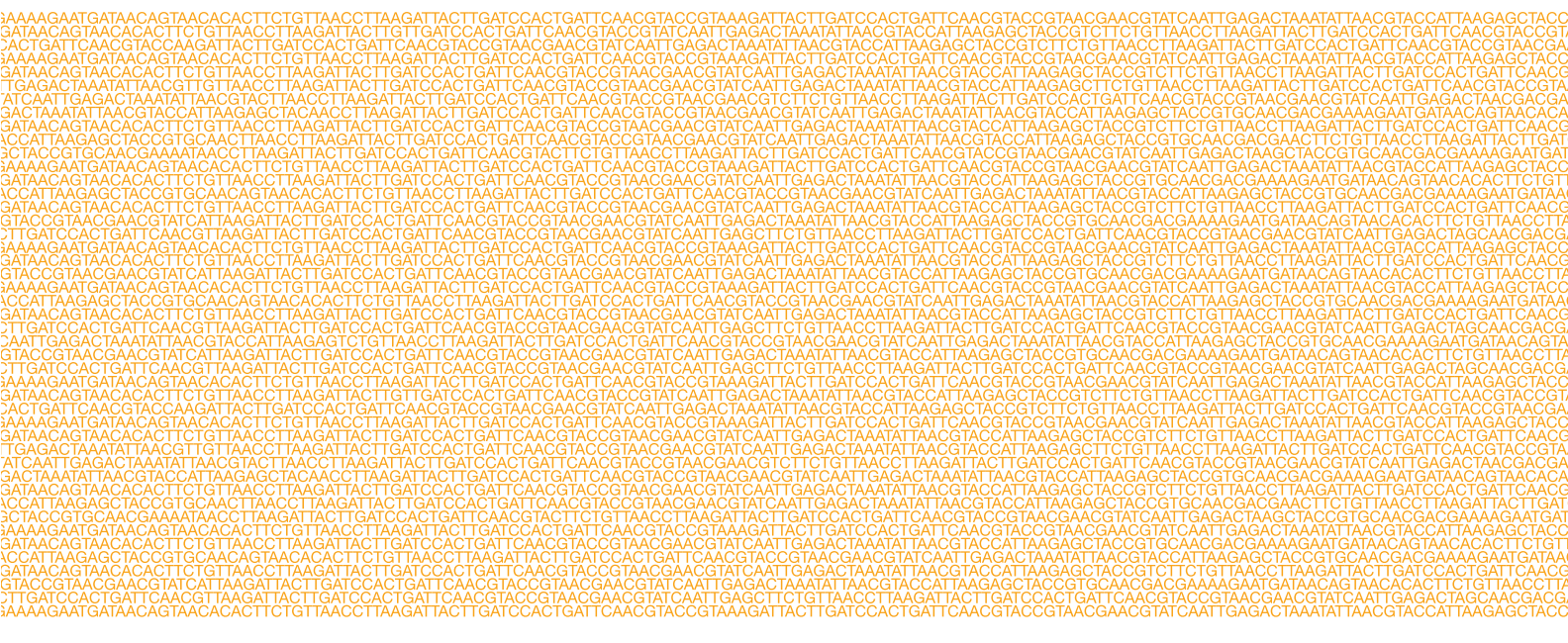
MSDS

Material safety data sheets (MSDSs) are available on the Illumina website at **http://www.illumina.com/msds**.

Product Documentation

If you require additional product documentation, you can obtain PDFs from the Illumina website. Go to **http://www.illumina.com/support/documentation.ilmn**.

When you click on a link, you will be asked to log in to My Illumina. After you log in, you can view or save the PDF. To register for a My Illumina account, please visit **https://my.illumina.com/Account/Register**.



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