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Eco™ Real-Time PCR System User Guide

FOR RESEARCH USE ONLY

ILLUMINA PROPRIETARY
Catalog # EC-900-1001
Part # 15017157 Rev. G
July 2013

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 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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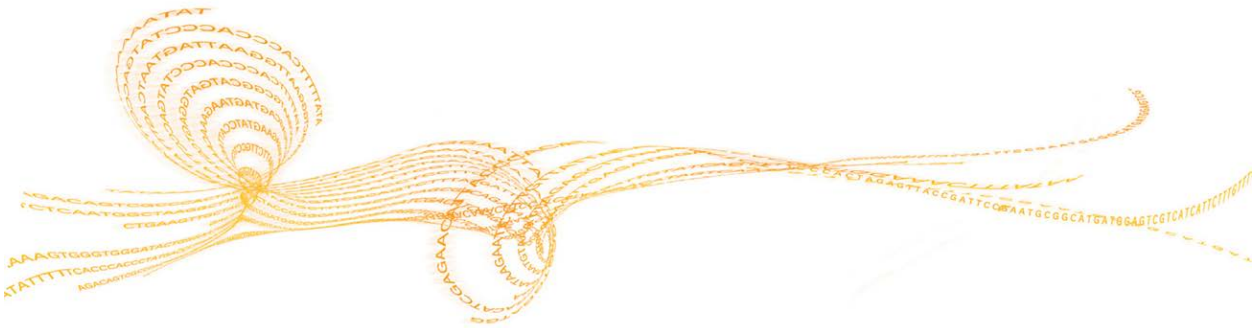
Revision History

Part #	Revision	Date	Description of Change
15017157	G	July 2013	Added the following information: • NuPCR detection chemistry • Using the New Session tab • Troubleshooting • Assays and reporter dyes for genotyping • System requirements Updated information on Eco-compatible dyes. Updated information on how to define a new experiment for genotyping and HRM experiments. Updated error light information. The data analysis function is removed from v5 of the Eco software. As a result, Analyze Data sections are removed and are now in the *EcoStudy User Guide (part # 15030781)*.
15017157	F	Feb 2012	Added information on baseline correction. Added information on setting up assays for genotyping experiments. Updated Eco-compatible dye information.
15017157	E	July 2011	Added instructions on how to open the instrument lid. Updated instrument-to-computer connections information.
15017157	D	May 2011	Corrected electrical and temperature range specifications.
15017157	C	Jan 2011	Added setup instructions. Added information on High Resolution Melt (HRM) experiments. Added information on relative quantification formulas.
15017157	B	August 2011	Replaced plot data graphics.
15017157	A	July 2010	Initial release.

Table of Contents

Overview

Chapter 1



Introduction

The Eco 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, Cy5, and Q670.

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 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.

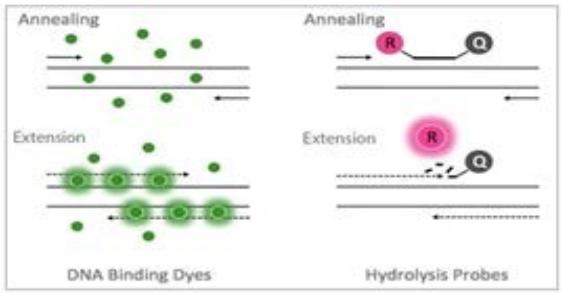
Real-Time PCR

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.

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.

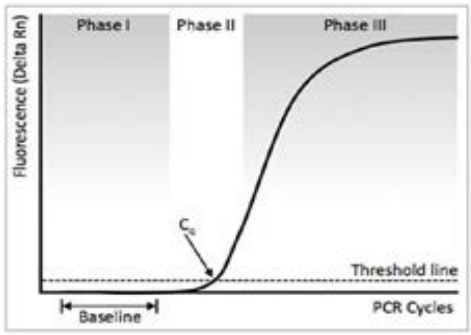
The two most commonly used qPCR chemistries are DNA binding dyes and hydrolysis probes (Figure 1). DNA binding dyes fluoresce when bound to double-stranded DNA. Hydrolysis probes fluoresce when the reporter molecule is removed from its quencher molecule by the 5′ exonuclease activity of DNA polymerase.

Figure 1 Main Real-Time PCR Chemistries



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).

Figure 2 The Three Phases of qPCR



Resources

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

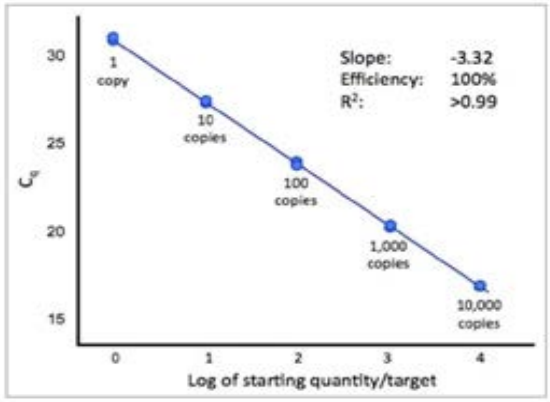
Higuchi R, Fockler G, Dollinger G, and Watson R (1993) Biotechnology (N.Y.) 11: 1026–1030

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

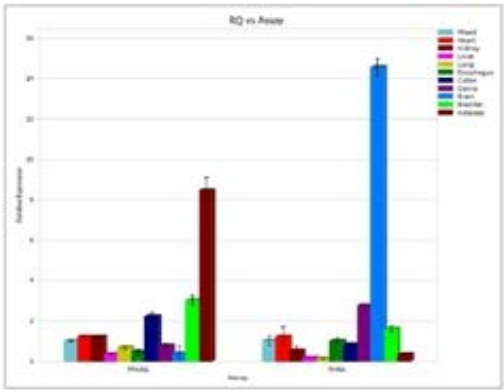


The slope of the standard curve measures the efficiency of the assay ($E = 10^{[-1/slope]} - 1$). A slope outside the acceptable range (slope -3.1 to -3.6 and E value between 90 and 110%) typically indicates a problem with the template or assay design. The R^2 value, a measure of reaction performance,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

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).

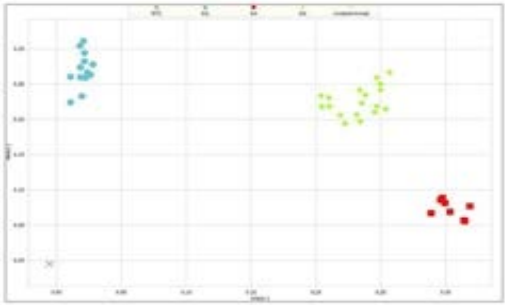
Figure 4 Relative Quantification Experiment



Genotyping and High Resolution Melt

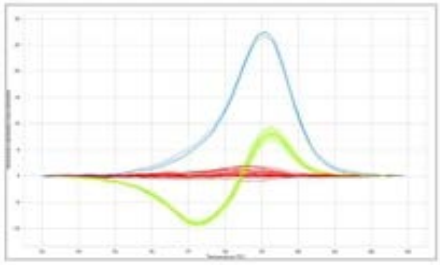
Genotyping (allelic discrimination) assays using hydrolysis probes provide a rapid and sensitive method to genotype samples. These assays can refer to a single nucleotide polymorphism (SNP) or insertion/deletion assays. Two variants/alleles are interrogated at the same time (multiplex qPCR). Most frequently, 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 Distribution 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.

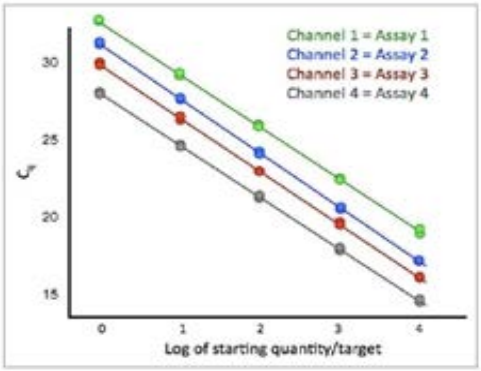
Table 1 Examples of Eco-Compatible Dyes

Channel	Dye
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], Q670[a]

a. Factory-Calibrated Dyes

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 Standard Curves for Four Multiplexed Assays

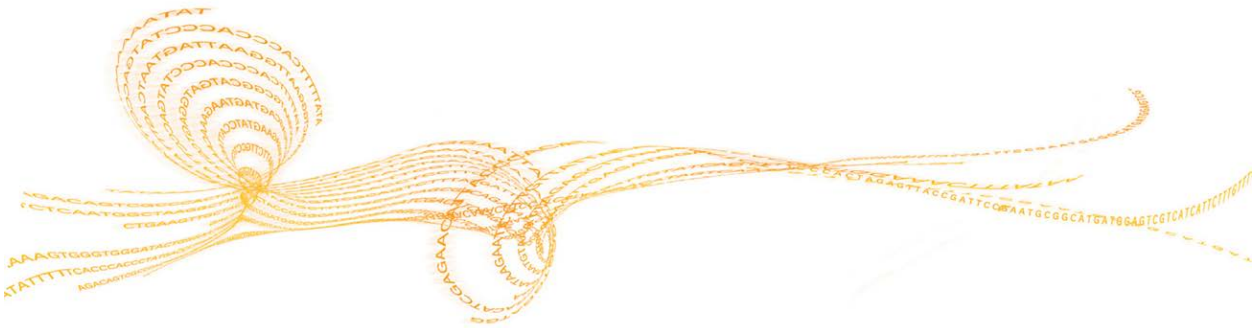


The Eco includes two excitation LED arrays (452-486 nm and 542-582 nm) and four filter channels (Table 1), which enable detection of up to four separate targets in a single reaction (Figure 7).

Eco is factory-calibrated for certain dyes within each channel (marked in Table 1), but also supports additional dyes that are excited and detected within the instrument specifications.

Setup

Chapter 2



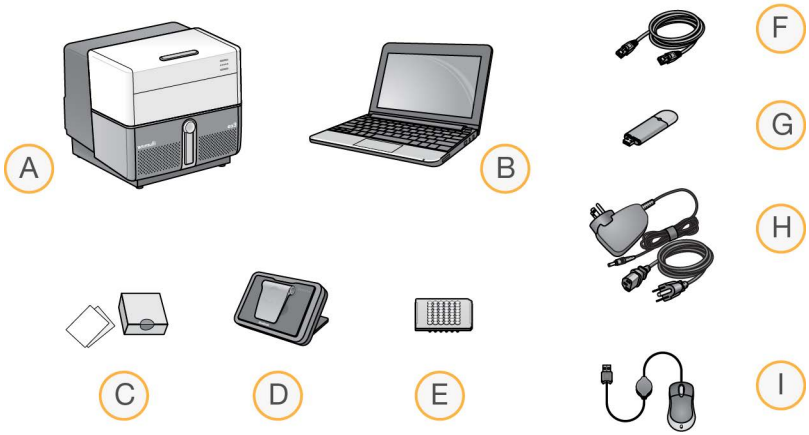
Unpack the Eco

1 Remove the computer and accessories from the box.

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.

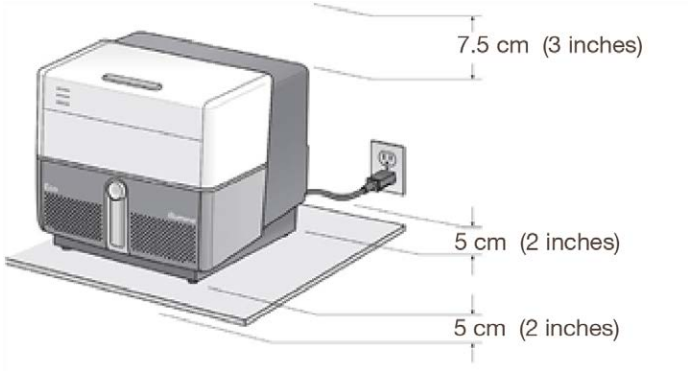
3 Check to ensure that all components are present and intact.
Your system comes with:



A Eco instrument
B 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 Mouse

Place Eco on the Bench

Figure 8 Eco Space Requirements

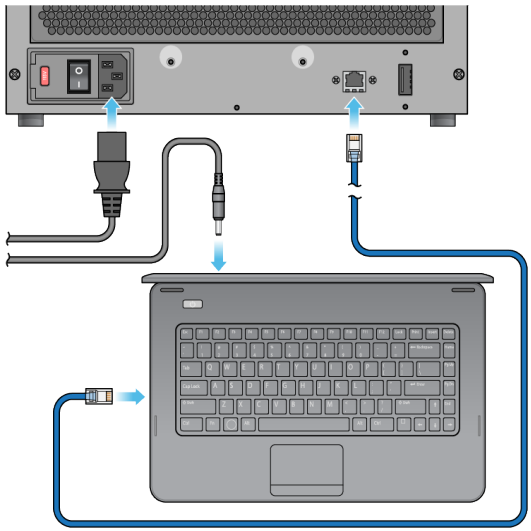


Eco requires 5 cm (2 inches) of unimpeded space at the front and back for ventilation and 7.5 cm (3 inches) above the instrument so that the lid can be opened safely.

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 9 Eco Connections



1 Connect one end of the Ethernet cable to the Ethernet port on the computer. 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 computer power cord to the wall outlet (C).

Turn on the Eco

Figure 10 Eco Startup Sequence

1 Turn on the 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 computer and the Eco instrument is 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).

Register your Eco

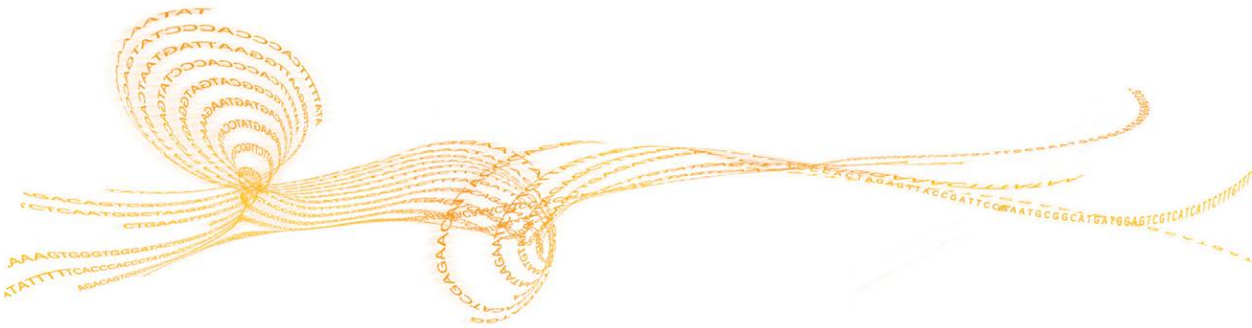
Once your Eco system is set up and ready to use, register your Eco by going to **www.illumina.com/registeryoureco** and completing a short questionnaire. Registering your Eco ensures that you will receive software updates in the future.

While you are visiting the web site, take advantage of the following online resources to support your research.

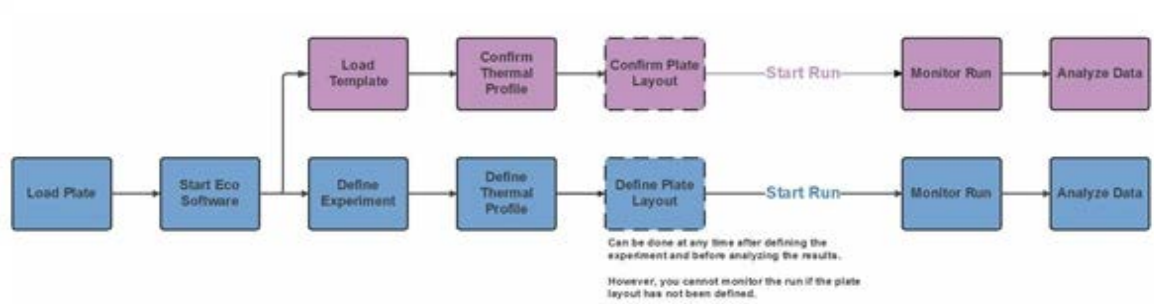
Eco Customer Support, knowledge database, warranty information, webinars, and seminar series	**http://support.illumina.com/ecoqpcr**
iCommunity *A quarterly e-newsletter about, for, and by the Illumina community*	**www.illumina.com/icommunity**
Online Ordering	**my.illumina.com**
Tradeshows, workshops, and meeting presence	**www.illumina.com/events**

Workflow

Chapter 3



Eco Workflow



1 Prepare the sample plate, load it into the Eco, and close the lid. See *Load the Plate* on page 19.

2 Launch the Eco software on the PC.

3 Define and name the experiment by selecting the application, detection chemistry, starting material, and specific method for your application. See *Define a New Experiment* on page 20.

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 computer.

4 Review the thermal profile and adapt it if needed. See *Set Up the Thermal Profile* on page 24.

5 Set up the plate layout by defining assays, samples, and standards and assigning them to wells. See *Define the Plate Layout* on page 26.

6 Start the run. The Monitor Run tab opens. See *Monitor Run* on page 38.

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 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.

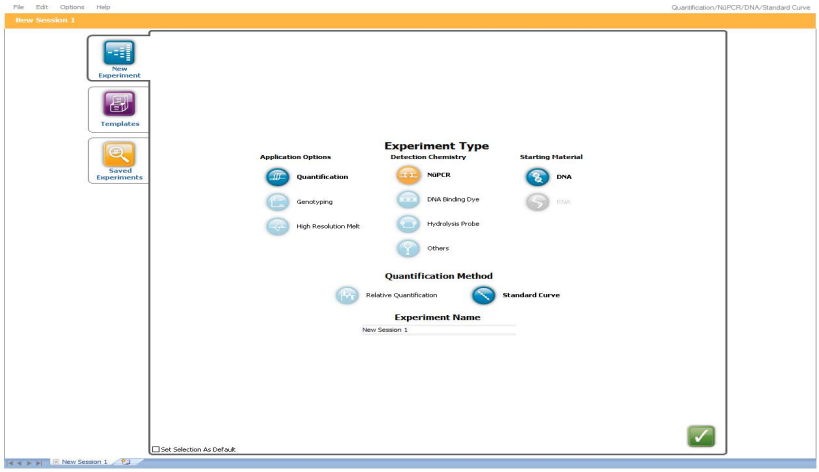
   WARNING
   Forcing the plate into any other orientation could damage the instrument.

   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 11 New Experiment Tab



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

2 Click an **Application Option** that is the specific method or protocol you want to use for your experiment.
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 three detection chemistries are grayed out for High Resolution Melt experiments.

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**

3 Select a **Detection Chemistry**.

Detection Chemistry	Type
NuPCR	See *NuPCR Chemistry* on page 21
DNA Binding Dye	SYBR green assays
Hydrolysis Probe	5' nuclease assays
Other	Non-hydrolitic assays

4 Select a **Starting Material**.

5 Enter an experiment name of up to 20 characters.

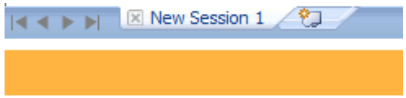
6 Click . The Setup window opens, with the Thermal Profile tab visible.

New Session Tab

Use the New Session tab to create, open, and select multiple experiments. The tab is on the bottom left of the screen. To add a new experiment, select the page with a star icon.

You can also right-click on the New Session tab to rename an experiment, make a new experiment, open an experiment, or close an experiment.

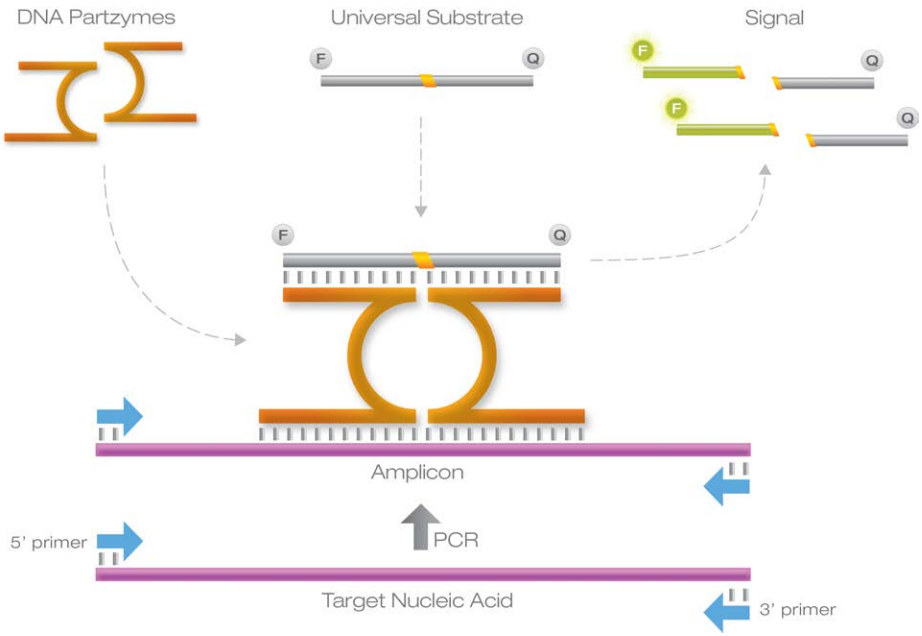
Figure 12 New Session Tab



NuPCR Chemistry

The NuPCR™ Assay PCR protocol can be used to amplify and detect any DNA target (genomic DNA (gDNA), complementary DNA (cDNA) or plasmid DNA). NuPCR Gene Expression assays are designed using Illumina® DesignStudio™ software and contain all the oligonucleotides required for performing nucleic acid enzyme, NuZyme™, based quantitative PCR (qPCR). The NuPCR Assay can be run on any real-time PCR instrument.

Figure 13 NuPCR Assay Concept



NuZymes are nucleic acid enzymes that recognize and assemble on target DNA sequences forming a catalytic complex. The enzymatic activity of the NuZyme cleaves a fluorescently-labeled universal substrate, producing a signal that can be detected by a real-time PCR instrument.

NuZymes are composed of two oligonucleotides that are partial enzymes or PartZymes™. Each PartZyme contains part of the catalytic core sequence of the NuZyme, flanked by a substrate arm sequence and a sensor arm sequence. Each sensor arm binds with high specificity to its target sequence, while the substrate arms bind the fluorescently-labeled, quenched universal substrate.

PartZymes contain no catalytic activity individually or in the absence of the target sequence. The target sequence serves to bring the two partzymes in close proximity, which facilitates the formation of the catalytically active NuZyme. The active NuZyme can now cleave the universal substrate oligo, however the target sequence remains intact. NuZymes are multiple-turnover enzymes, cleaving multiple substrate oligos in succession.

During a NuPCR reaction, target sequences are amplified using 5' and 3' primers and a DNA polymerase. The sensor arms of the partzymes bind to their corresponding sequences in the target amplicon, forming the catalytic NuZyme. The universal substrate binds the

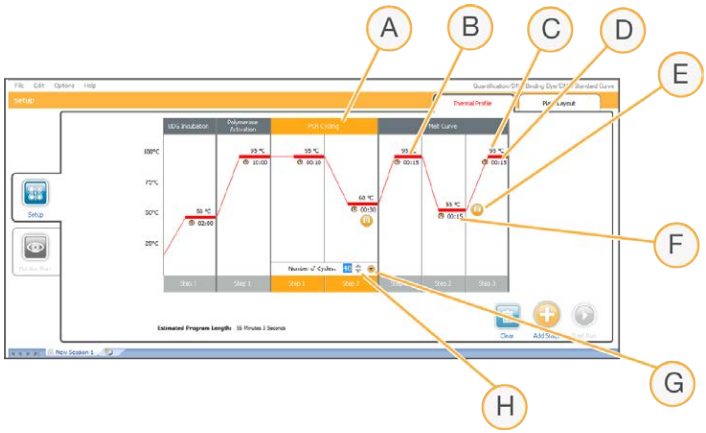
NuZyme substrate arms and is cleaved, releasing the fluorophore from the quencher, resulting in increased fluorescence. The fluorescent signal allows for detection and quantification of target sequences in real time.

NuPCR assays are supplied as a 20X solution containing all the oligonucleotides required for performing NuZyme based qPCR. The NuPCR master mix is provided as a 2X formulation containing all the components required for real-time PCR except primers, universal substrate, and DNA template. The ROX passive reference dye can be used for instruments that require it.

Multiplex PCR with NuPCR

Two-color multiplex real-time PCR can be performed with NuPCR. DesignStudio can design optimal primer and NuZyme sets for single tube multiplex assays for up to two targets. All sets are analyzed for oligo interactions and homology to other targets for specific and efficient amplification.

Set Up the Thermal Profile



- **A** Drag to move the stage
- **B** Double-click the temperature plateau to adjust temperature and duration
- **C** Type new temperature
- **D** Drag the bar up and down to adjust the temperature
- **E** Data collection point
- **F** Type new cycle time
- **G** Toggle two, three, four, or five step PCR
- **H** Click or type to add or remove cycles

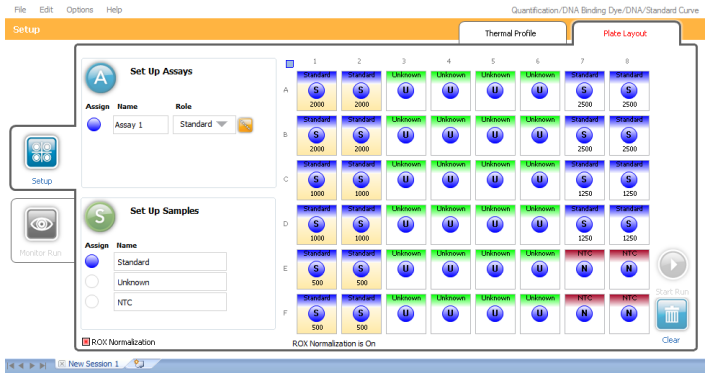
When you define the experiment a corresponding default thermal profile is selected automatically. You can use this or modify it based on your reagent's recommended protocol. You can set up cycle parameters in the Thermal Profile at any time after defining the experiment, but only before starting the 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. Only one step can be designated to collect image data.
- To remove a stage, drag it to the  trash can or highlight it and press **Delete**.

Define the Plate Layout

Figure 14 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. See *Set Up Assays* on page 29.

2 Set up samples. See *Set Up Samples* on page 32.

3 Assign assays and samples to wells. See *Assign Assays and Samples to Wells* on page 33.

4 Define standards (Standard Curve Quantification experiments only). See*Define Standards* on page 34.

5 Select the Rox Normalization checkbox if you are using Rox passive reference dye to normalize across your plate.

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.

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.

Table 2 Channels and Reporter Dyes

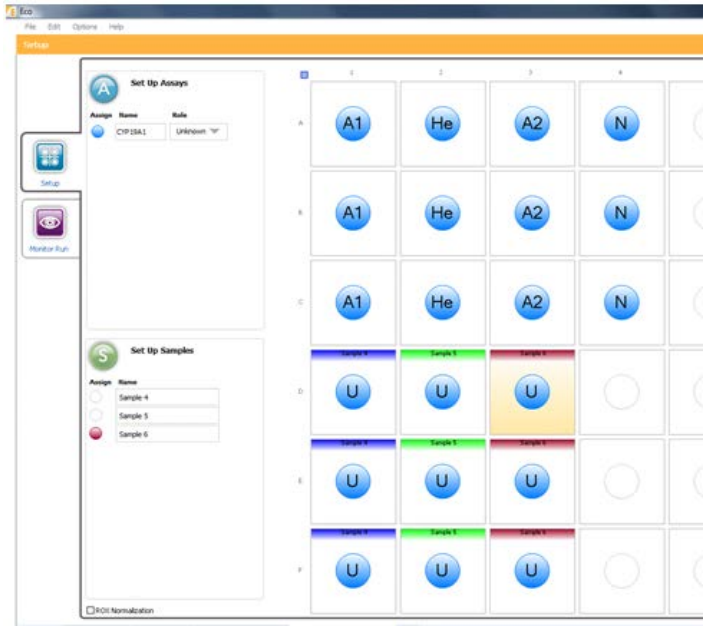
Channel	Excitation (nm)	Emission (nm)	Fluorophores Calibrated on the Eco (Reporter)
1	452-486	505-545	SYBR Green I, FAM
2	542-582	604-644	ROX[a]
3	452-486	562-596	HEX, VIC
4	542-582	665-705	Cy5, Q670

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.

Assays and Reporter Dyes for Genotyping

When defining a plate layout, genotyping experiments need special setup of assays and reporter dyes. Assign at least one well for each homozygous Allele 1, homozygous Allele 2, and for heterozygous controls. Make sure to select a different reporter dye for each Allele. See *Genotyping and High Resolution Melt* on page 7.

Figure 15 Example Plate Layout for a Genotyping Experiment



Set Up Assays

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

2 Use the arrow keys to select the number of assays.

3 For each assay:

- Define a name and color.
- For Genotyping experiments: In the **Allele 1 Base** and **Allele 2 Base** drop-down list, select **A, C, G** or **T** if the assay refers to a SNP. For insertion or deletion assays, select **INS** or **DEL**.
- 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 *Multiplexing Real-Time PCR* on page 9).
  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.

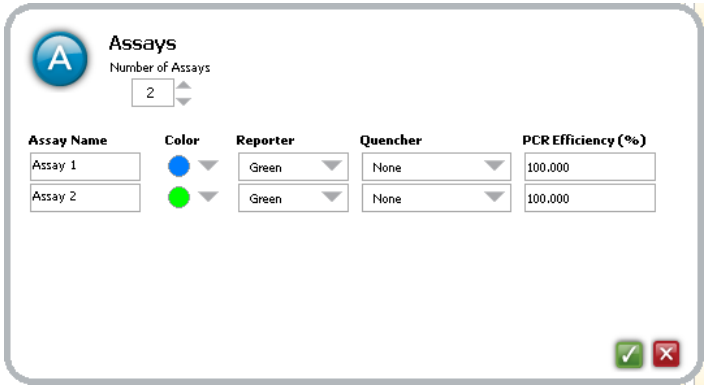
NOTE
BHQ and MGB are considered non-fluorescent quenchers.

NOTE
Fluorescent quenchers such as Tamra are not recommended for use in the Eco.

  For Genotyping experiments: Select a quencher for Alleles 1 and 2.
- For Relative Quantification experiments: Specify the **PCR Efficiency (%)**.

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

Figure 16 Assay Dialog Box, Relative Quantification Experiments



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

6 For all experiment types: If you want to use controls, select a control type for each assay from the Role drop-down list. Options for roles in the drop-down list change according to the type of experiment you do.

NOTE
The Role you assign has no affect on the analysis calculations of your experiment. The Role is just a label for your convenience. For example, if you want to define an assay as a no reverse transcription control, or you want to define an assay as a control sample that you know to be negative for the target you are amplifying, you can select the Role "Negative" or "NTC". Data from the negative control or NTC is not used in calculations to normalize the data.

7 For Genotyping and High Resolution Melt experiments: If you want controls with unique names that are not included with the software, set them up from the Options menu. After controls are created, they are available for use in the Role drop-down list in the Assays section.

   a Click the **Options** menu.

   b Click **Control Types**.

   c Use the arrows to select the **Number of Control Types**.

   d Select the **Color**.

   To change the color, select the round color icon next to the assay name. Double-click a new color in the color palette.

   e An **Abbreviation** automatically populates.

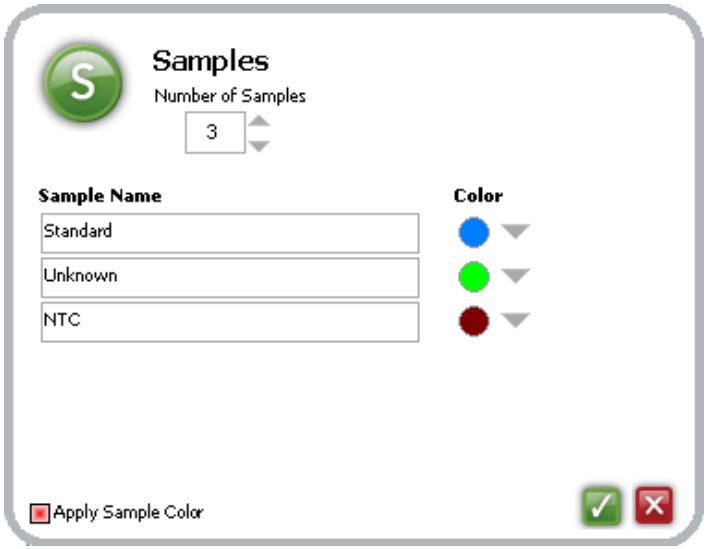
   f Click **OK**  .

8 Proceed to set up samples.

Set Up Samples

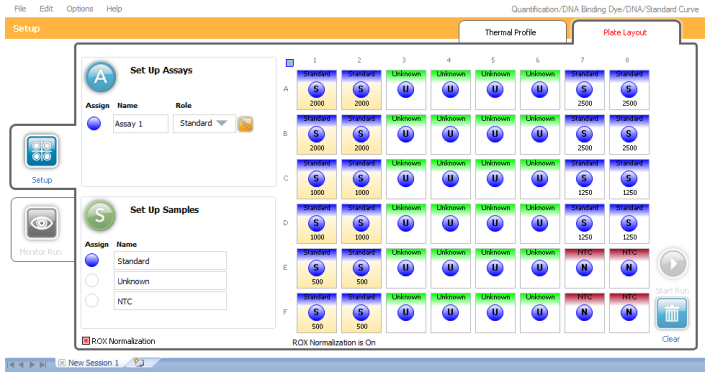
1. On the Plate Layout tab, click or **Set Up Samples** to open the Samples dialog box.
2. Use the arrow keys to select the number of samples.
3. For each sample, define a name and color.
4. Click to close the Samples dialog box and return to Plate Layout.
5. For Relative Quantification and HRM experiments: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

Figure 17 Sample Dialog Box



Assign Assays and Samples to Wells

Figure 18 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 yellow when they are highlighted, as shown in columns 1 and 2 of Figure 18.

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

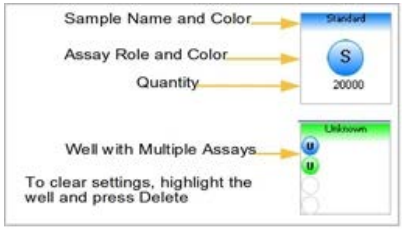
- Standard Curve studies: At least two wells with the role "Standard", but with different quantities
- Relative Quantification studies: At least one well with the role "Unknown" or "Positive" and a sample assigned

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.

Figure 19 Well in the Plate Layout



Define Standards

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

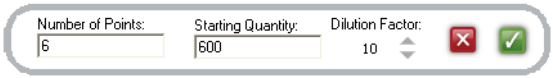
1 Click to open the Set Up Standards pane in the lower left of the window.

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

Figure 20 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

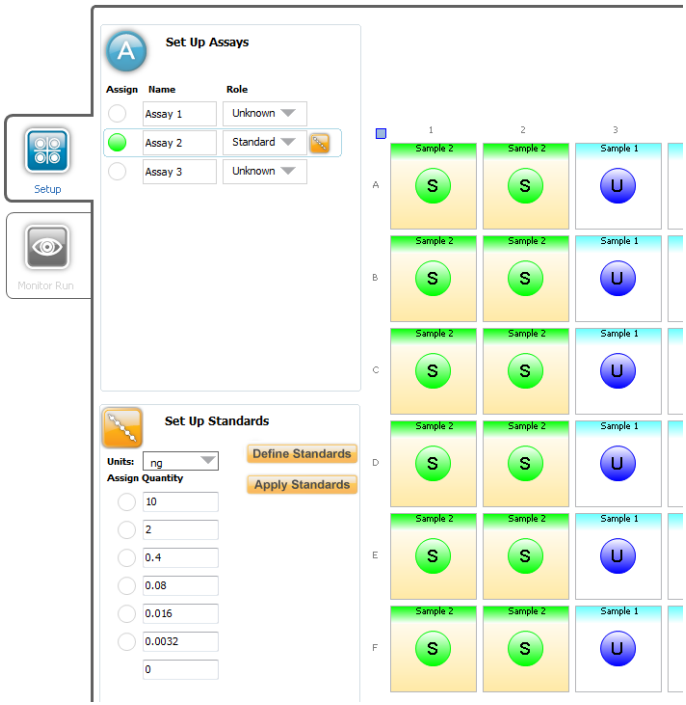
You can assign standard dilutions to wells manually or automatically.

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.

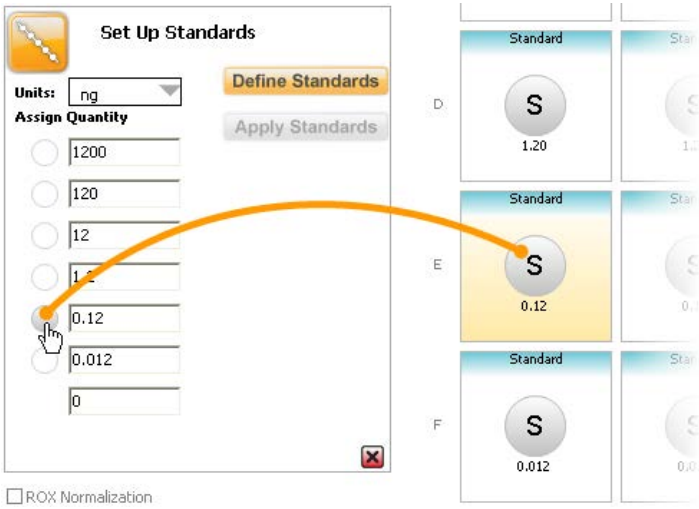
Figure 21 Selecting Standard Assay Wells



Assign Dilutions Manually

Highlight a Standard Assay well and click the **Assign** button beside the appropriate dilution quantity (Figure 22).

Figure 22 Assigning Dilutions



Monitor Run

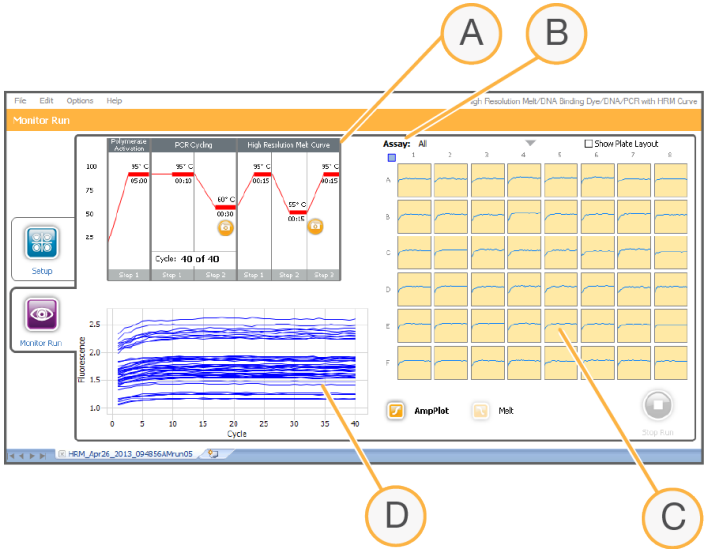
WARNING

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 (*Define the Plate Layout* on page 26), you will only be able to view progress against the Thermal Profile on this tab.

Figure 23 Monitor Tab



A Select assays to view in Amplification Plot
B Shows current stage of the Thermal Profile highlighted in orange
C Amplification View shows deconvoluted data in real time for each well
D Amplification Plot shows deconvoluted data in real time for selected wells

Figure 24 Amplification Plot

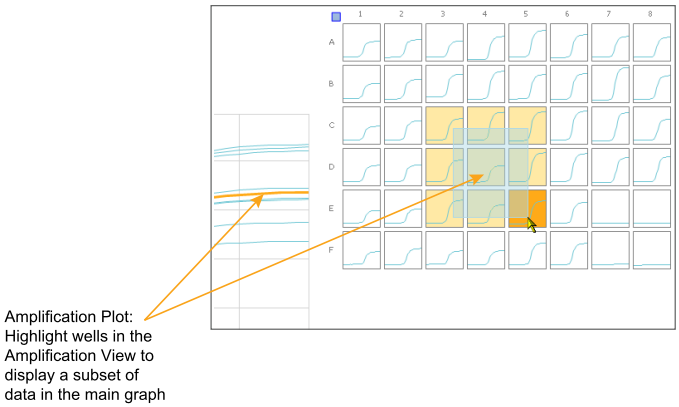
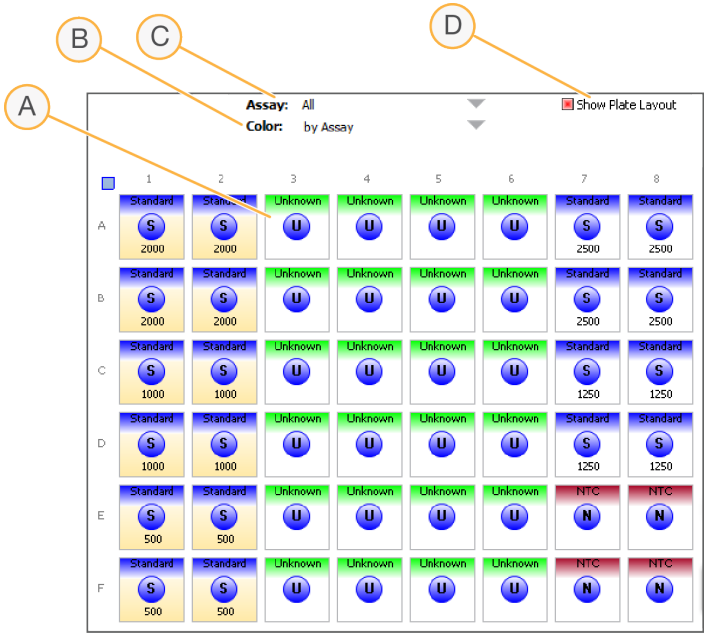


Figure 25 Plate Layout View in the Monitor Tab

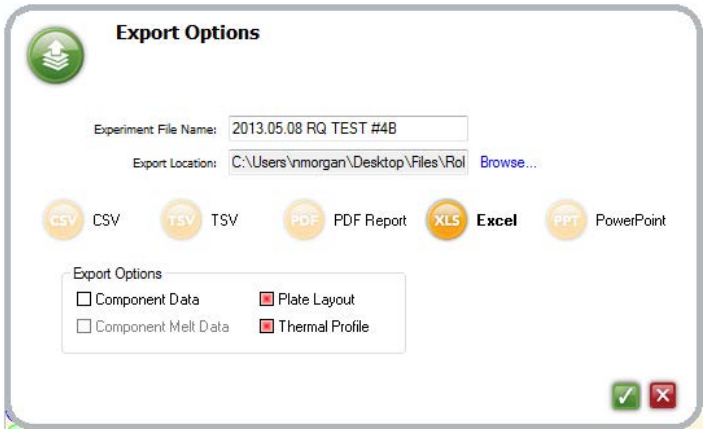


A Plate layout view shows sample type, sample identity, dilution and assays
B Select by assay, sample or call using the drop-down arrow
C Select the identity of the assay using the drop-down arrow
D Turn Show Plate Layout on and off by selecting the box

Export Results and Data

Results, as well as other data, can be exported by selecting **File** | **Export** in the main menu. The options available here vary depending on the type of experiment you ran and the file format you chose to output. If a type of data is not available, it appears grayed out in the Export Options area. In the Export Options dialog box, select the desired file format and components to export, then click .

Figure 26 Export Options dialog box



Templates and Sample Sheets

The table below explains the differences between a template, a plate template, and a sample sheet. It also describes where and when templates and sample sheets can be saved, imported and exported. The commands below are available under the File menu in Eco. Only Import Plate Template and Export Plate Template are available in EcoStudy.

Command	Save a Template	Import a Sample Sheet	Import Plate Template	Export Plate Template
Description	A template is a plate layout and thermal profile.	A sample sheet is a list of assays and samples, but unassigned to a plate layout.	A plate template is a plate layout, and does not include a thermal profile.	A plate template is a plate layout, and does not include a thermal profile.
File Extension	*.ecot	*.csv	*.csv	*.csv
Where	Eco	Eco	Eco or EcoStudy	Eco or EcoStudy
When	Before a run	Before and after a run, but not during a run	Before and after a run, but not during a run	Before and after a run, but not during a run
Notes	After saving, the template is available for use. It is listed in the Templates tab on startup in Eco.	See directions for Making a Sample Sheet for Import.	The Plate tab must be active.	

.

Making a Sample Sheet for Import

To create your own sample sheet for import into Eco, use a program like Excel to create a *.csv file. The sample sheet can contain up to 99 assay names and 48 sample names.

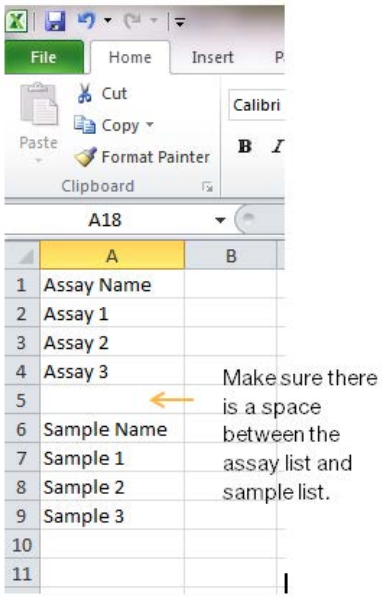
3 In the same column, enter the heading “Assay Name” and “Sample Name”.

4 Make sure there is a space between the assay list and the sample list.

5 Give each assay and sample a unique name.

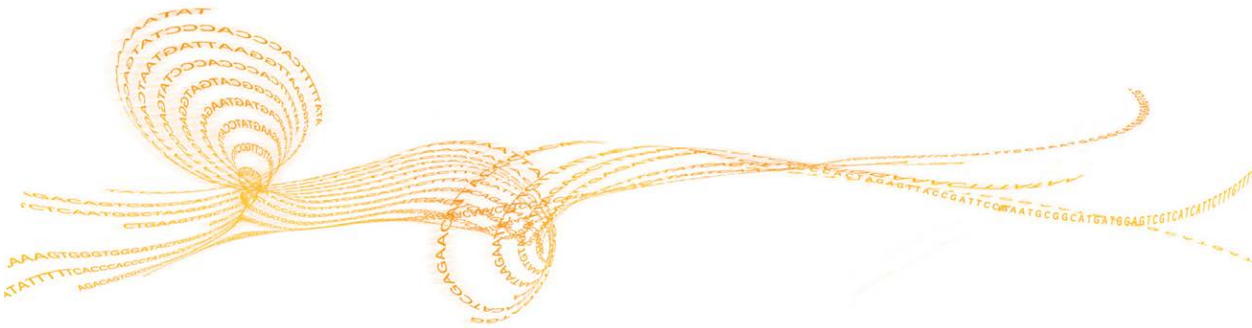
6 Save the file as a *.csv file.

Figure 27 Example of a Sample Sheet



System Information

Chapter 4

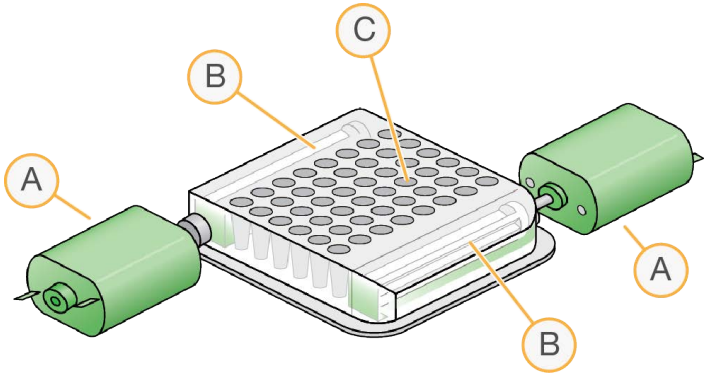


Components

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 28 Eco Thermal System



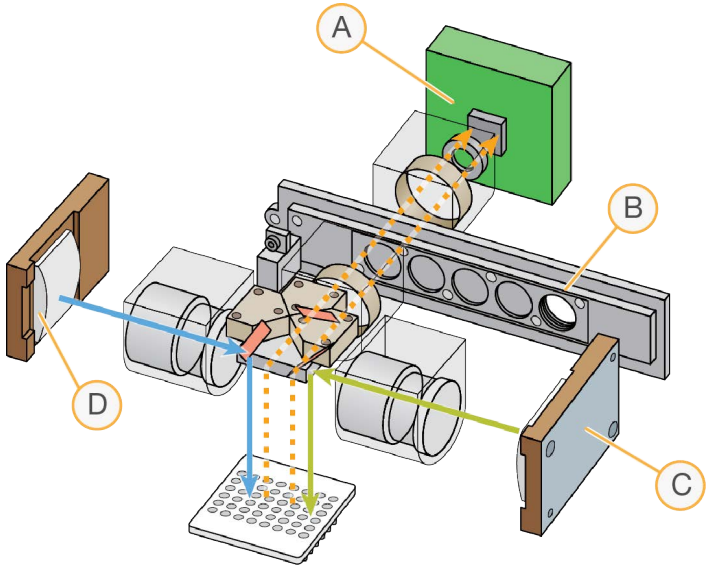
A Motors
B Stirring Paddles
C Silver hollow Thermal Block, containing the conductive fluid

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, Cy5 and Q670 dyes. You can also use other dyes that are compatible with the excitation and emission wavelengths.

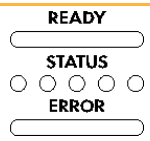
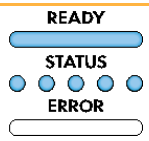
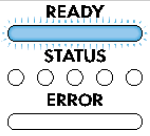
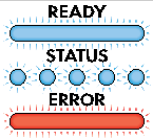
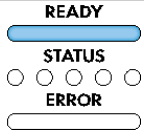
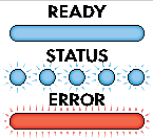
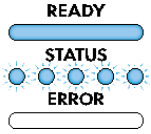
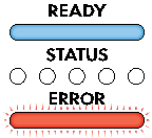
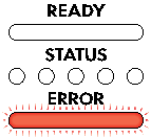
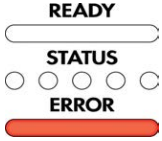
Figure 1 Optical System

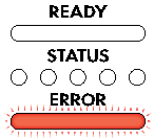
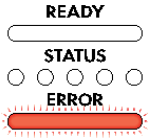
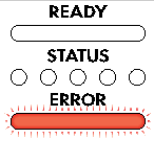
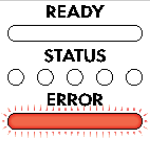


A CCD Camera
B Filter Slide
C Green LED Array
D Blue LED Array

Lights

The Eco has three indicator lights on the front: **Ready**, **Status**, and **Error**. The following tables show the meaning of each combination of off, on, and flashing lights. If Power Cycling is advised for an error light, see *Power Cycling* on page 56.

Lights	Description	Lights	Description
	Power Off		Run Complete
	Initializing (Conducting self tests and heating the thermal block).		Communicating with the Computer
	Ready/Idle		Software Updating
	Run In Progress **Do not switch off or open the lid while a run is in progress.**		Non-Fatal Error Decide whether you want to terminate the run.
	Fatal Error: Run Terminated Error light flashing. Instrument may have overheated or encountered a hardware failure.		Hardware Failure Solid Error light. Hardware problem. Call customer support.

Lights	Description	Lights	Description
	Camera Initialization Error 2 long flashes of the Error light. Try power cycling the instrument.		Camera Trigger Error 3 long flashes of the Error light. Try power cycling the instrument.
	Temperature Sensor Error 4 long flashes of the Error light. Hardware problem. Call customer support.		Temperature Response Error 5 long flashes of the Error light. Hardware problem. Call customer support.

System Requirements

The following are system requirements for running Eco software:

- Operating system must be Windows 7 only, 32 or 64 bit
- Memory (RAM): ≥ 1 GB
- CPU: ≥ 1.6 MHz, 64 bit processor

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
	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.

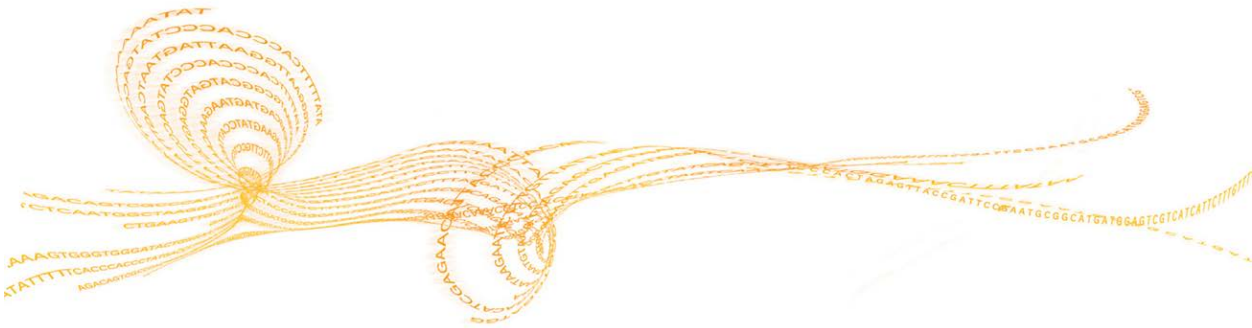
Follow these practices for proper maintenance of your Eco.

- 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 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.

Troubleshooting

Chapter 5



Introduction

For most errors on the Eco, an on-screen message opens with instructions for correcting the error. Error lights on the front of the instrument may also indicate a problem and how to fix it. See *Lights* on page 47.

If connection is lost between the Eco instrument and the computer, use the Run Recovery section to recover your run data. See *Run Recovery* on page 56.

This Troubleshooting section also has directions on how to access files and information that will help Illumina Technical Support troubleshoot your problem. For instance, if you contact an Illumina Technical Support representative, they might ask for copies of run-specific files for troubleshooting purposes, such as a log file.

For technical questions, visit the Eco support pages on the Illumina website for access to frequently asked questions, or log in to your MyIllumina account for access to support bulletins.

For problems with run quality or performance, contact Illumina Technical Support. For more information, see *Technical Assistance* on page 67.

Run Recovery

If the Eco instrument loses connection with the computer, the Eco often continues to run. After you reconnect your computer with the Eco, you can usually retrieve the run data because the data file of your last experiment is stored in the instrument.

To recover the run data, follow the Power Cycling directions below to turn the computer and the Eco off and on. After Power Cycling, select **Options | Recover Last Experiment** in the main menu.

Power Cycling

1. Turn off the power on the Eco. The power switch is on the back of the instrument.
2. Shut down the computer so that the power is off.
3. Wait three minutes.

NOTE
Make sure that the Eco and the computer are powered off for three minutes before you proceed to step 4.

4. Turn on the Eco instrument.
5. Turn on the computer.
6. Double-click the Eco icon on the computer desktop to start the Eco software. Communication between the computer and the Eco instrument is established within five minutes.

NOTE
If you are power cycling Eco because the Eco instrument and the computer lost connection, a warning message might appear during the connection time that says the experiment file may not have completed. The message asks you if you want the software to attempt to recover the run. Click **Yes**.

7. When **Status: Instrument Connected** displays at the bottom of the screen, the computer and the instrument are connected.
8. Wait until the Ready light on the Eco instrument is solid blue. This takes up to twenty minutes.

Recover Last Experiment

After you follow the directions for Power Cycling, you can recover the experiment that was running when the connection was lost between the Eco Instrument and the computer.

1. Select **Options** | **Recover Last Experiment** in the main menu.
2. The name of your latest experiment with the word "recovered" appears in the New Sessions tab at the bottom of the screen.
3. To confirm that your data is recovered, go to the Monitor Run tab. If your data is recovered, you will see the data in the Amplification Plot and the Amplification View.
4. Select **File** | **Save As** to save your recovered data.

Accessing Log Files

Eco stores a log file that helps with many troubleshooting issues. Illumina Technical Support may ask for this file. To access the log file, follow the procedure here.

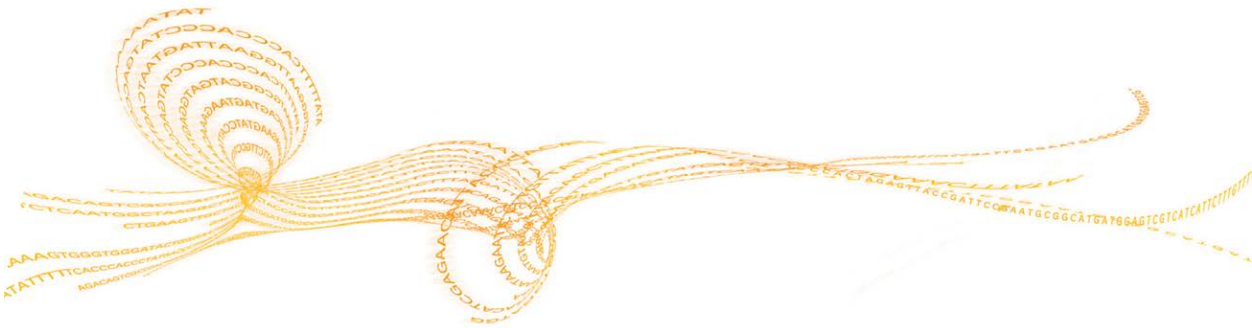
1. Turn on the Eco and the computer.
2. Double-click the Eco icon on the computer desktop to start the Eco software. Communication between the computer and the Eco instrument is established within five minutes. When **Status: Instrument Connected** displays at the bottom of the screen, the computer and the instrument are connected.
3. Close the Eco software on the computer.
4. Navigate to C:ProgramFiles\Illumina\Eco and find the GetLogs.exe file. *If you did not install the Eco program on the C drive, your drive letter may be different.*
5. Open the **GetLogs.exe** file.
6. Select the **Include Previously Uploaded Logs** check box.
7. Click **Get Log Files**.
   When the file finishes transferring, Done appears on the GetLogs dialog box.
8. Save the zipped log file on your computer in an easy-to-remember place.
9. Email the zipped log file to Illumina Technical Support at techsupport@illumina.com.

Identifying Serial and Version Numbers

To identify the Eco instrument ID (serial) number, the software version number, and the instrument firmware version number, select **Help | About Eco/License Information** on the main menu.

Concepts and Glossary

Appendix A



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) = (A260 * 40 * D)/1000, where D = dilution factor and A_{260} = absorbance at 260 nm.

DNA concentration (µg/µl) = (A_{260} * 50 * D)/1000, where D = dilution factor and A_{260} = absorbance 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 select are stable in your experiments.

Glossary

Term	Definition
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.

Term	Definition
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 spiked into each sample at a known concentration.
End-Point Analysis	Qualitative analysis of PCR data at the end of PCR. Allelic discrimination assays (genotyping) are an example.
Exogenous Control	An 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.

Term	Definition
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.

Term	Definition
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.

Term	Definition
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.
TAMRA	Tetramethyl-6-carboxyrhodamine. Commonly used as a quencher.
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).

Technical Assistance

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

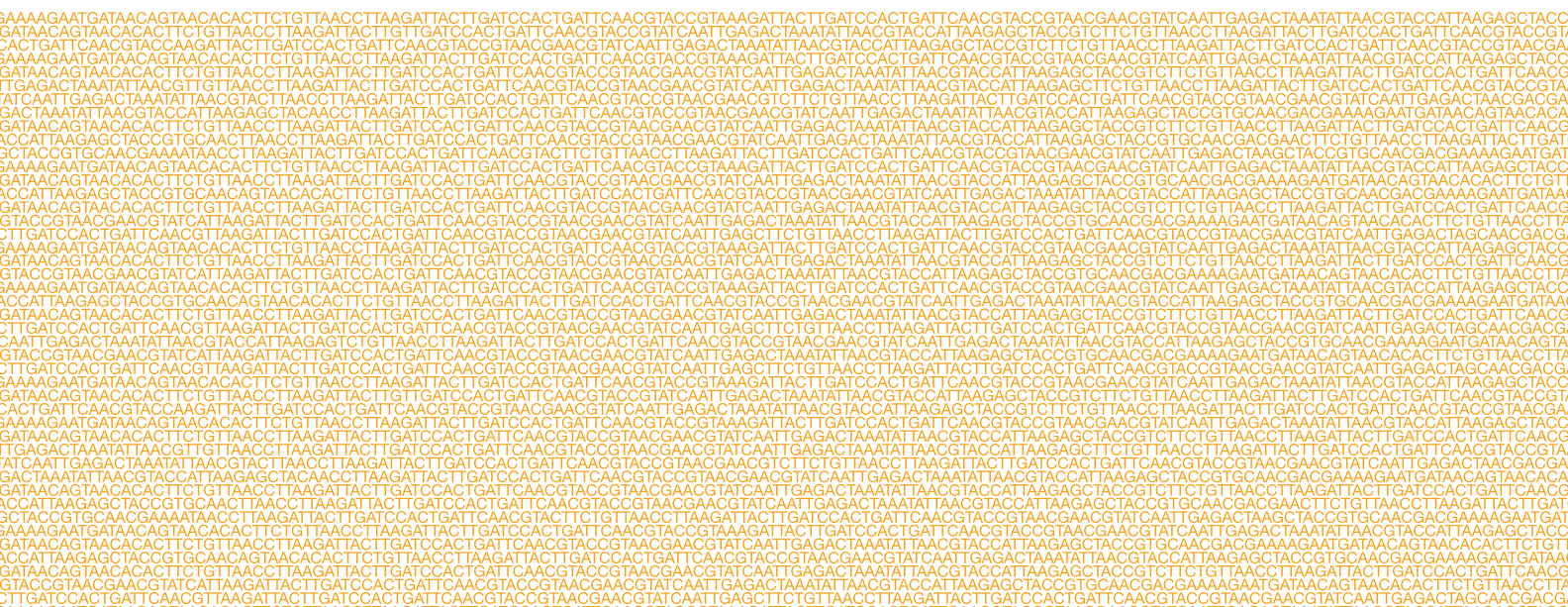
MSDSs

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 are 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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