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EcoStudy User Guide

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Part # 15030781 Rev. B
July 2013

Revision History

Part #	Revision	Date	Description of Change
15030781	B	July 2013	Added information on the following: • System requirements • Templates and sample sheets • Setting up the plate layout and analyzing experiment data • The calculation of Cq standard mean, standard deviation, standard variation, and standard error Added an appendix of Concepts and a Glossary. Updated the document throughout to reflect changes in the user interface. Updated instructions for exporting data. Updated the following information to include Genotyping and High Resolution Melt experiments: • Study requirements • Study analysis • Reviewing study data Updated instructions on setting up a study to include genotyping.
15030781	A	February 2012	Initial release.

Table of Contents

Overview

Chapter 1

What is EcoStudy?

The EcoStudy software is the data analysis component of Eco. EcoStudy enables you to analyze a run on Eco, and combine data from one or more Eco experiments into a study for analysis.

Multiple Experiment Analysis (MEA), which is the analysis of data from multiple plates, is supported for Standard Curve, Relative Quantification, and Genotyping experiments. Single experiment analysis is supported for High Resolution Melt (HRM).

NOTE
Illumina does not recommend combining experiments from multiple instruments because differences in raw fluorescence values between instruments might increase the systematic error in your measurements.

NOTE
HRM (High Resolution Melt) experiments cannot be combined for analysis in EcoStudy.

System Requirements

The following are system requirements for running EcoStudy software:

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

Opening EcoStudy

When you start the EcoStudy software, a startup screen opens. The startup screen is divided into three sections; Start New Study, Open Existing Study, and Recent Locations.

Start New Study

Click **Add Experiment** to open a new experiment, or to open an experiment from a list of recent experiments.

Open Existing Study

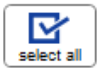
Click **Open Study** to open a study created before, or to open a study from a list of recent studies.

Recent Locations

Click **Recent Locations** to navigate to folders that contain experiments or studies.

EcoStudy Icons

Table 1 EcoStudy Icons

Icon	Icon Name	Function
	Settings	Use for analysis settings in experiments and studies. The settings button functions in all tabs.
	Export	Use to export data in *.csv, *.tsv, Excel, and PowerPoint formats. Functions in all tabs.
	Auto Scroll	Use in the Amplification Plot tab, Melt Curve tab, and Results tab. With Auto Scroll active, anything you select in a graph will scroll automatically to select the information in the corresponding entry in the well table.
	Selection	Use in Genotyping and HRM studies and experiments. Click the Selection button. Then, use your mouse to select an area of interest in the Amplification Plot tab. For HRM, use your mouse to draw a line on parts of the curve to select. For genotyping, draw a polygon around clusters of interest. The area you select will turn orange. Use in the Amplification Plot tab, Melt Curve tab, and Results tab.
	Select All	Click in either the graph or the well table to select everything in it. To undo Select All, click outside of the well table or graph you selected, and then click back into it. Use in the Amplification Plot tab, Melt Curve tab, and Results tab.
	Undo Zoom	To zoom over an area of interest in a graph, hold down the left mouse button and drag. Use the Undo zoom button to return to the full view of the graph. Use in the Amplification Plot tab, Melt Curve tab, and Results tab.
	Clear Filter	Use to clear any filters set in the well table. Functions in the Amplification Plot tab, Melt Curve tab, and Results tab.
	Apply / Unapply	Use to apply or remove assay, sample or quantity information on wells. For the button to work, a well must be selected. Functions in the Plate Layout tab.

Exporting Data from EcoStudy

You can export results and other data from your experiment or study in a variety of formats, including comma- and tab-delimited text files, a Microsoft Excel spreadsheet, and a Microsoft PowerPoint presentation.

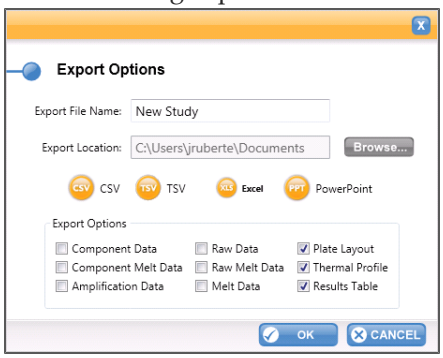
1 Click the **Export** button, or select **File** | **Export** in the main menu.

2 Type a name for the export file.
The Export File Name field is pre-populated with the name of the study. If you did not select a name for the study, New Study is displayed.

3 Click **Browse** next to **Export Location** and navigate to the folder where you want to save the export data.

4 Select the data export format by clicking a yellow button above the Export Options area.

5 In the **Export Options** area, select a check box for each type of data you want exported. The options available here vary depending on the type of study 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 this example, the Plate Layout, Thermal Profile, and Results Table are being exported to Excel.



6 Click **OK**.
Your exported data opens in the appropriate software application for the file format you chose to export.

Setting Up the Plate Layout

Chapter 2

Define the Plate Layout

The Plate Layout tab lets you define how your samples, assays, and standards are laid out on the plate loaded in the Eco. EcoStudy uses the plate layout to calculate data values. The Plate Layout tab is available in both Eco and EcoStudy.

Figure 1 Plate Layout Tab

Plate layout involves the following steps:

1 Set up assays. See *Set Up Assays* on page 11.

2 Set up samples. See *Set Up Samples* on page 13.

3 Assign assays and samples to wells. See *Assign Assays and Samples to Wells* on page 14.

4 Define standards (Standard Curve Quantification experiments only). See *Define Standards* on page 15.

5 Select Rox Normalization check box 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 in Eco.

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 yellow triangle with an exclamation point in 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

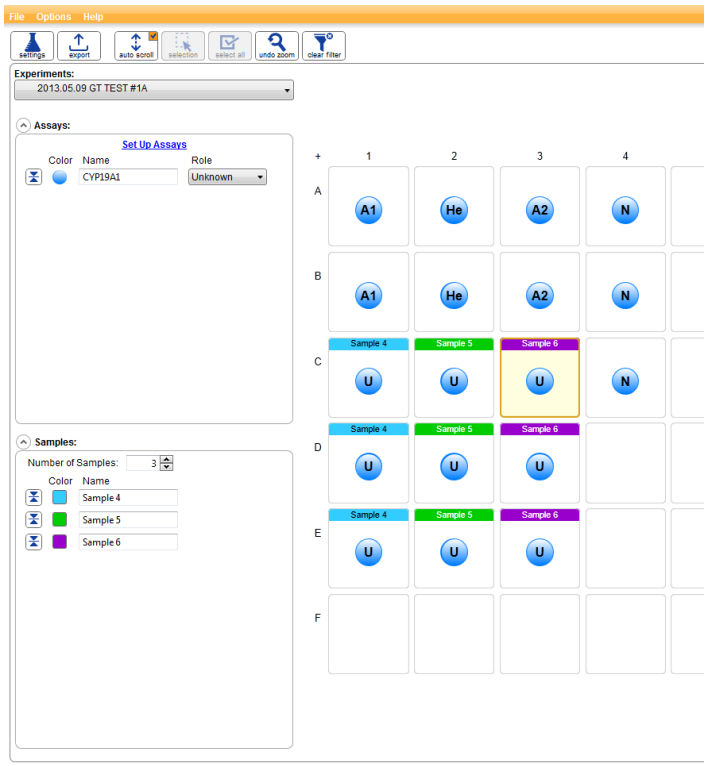
Channel	Excitation (nm)	Emission (nm)	Fluorophores Calibrated on the Eco (Reporter)
1	452-486	505-545	SYBR Green I, FAM
2	542-582	604-644	ROX[a]
3	452-486	562-596	HEX, VIC
4	542-582	665-705	Cy5, Q670

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 (allelic discrimination) 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. For more information about genotyping experiments, see *Eco Real Time PCR User Guide*, Part # 15017157.

Figure 2 Example Plate Layout for a Genotyping Experiment



Set Up Assays

1 On the Plate Layout tab, click **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.
- Define a color.
  To change the color, click the round color icon next to the assay name. Click a new color on the color palette.
- 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. See *Channels and Reporter Dyes* on page 9.
  For Genotyping experiments: Select a reporter dye for Alleles 1 and 2.
  For HRM experiments: Select the reporter dye SYBR **Green**.
- 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.

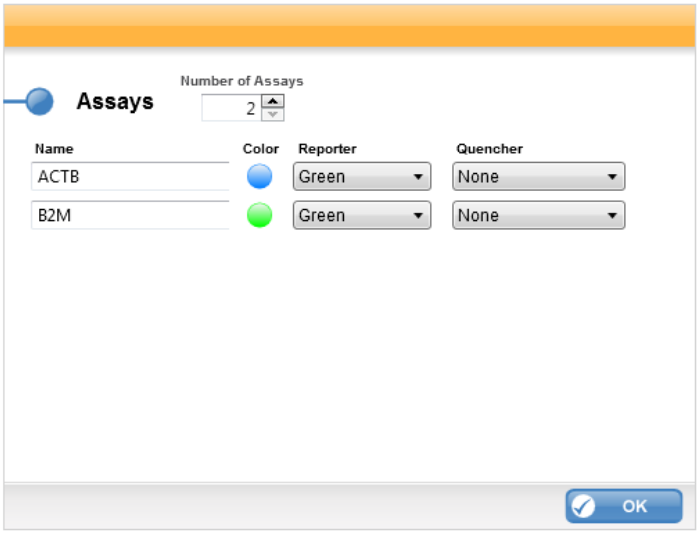
NOTE
BHQ and MGB are considered non-fluorescent quenchers.

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.

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

Figure 3 Assay Dialog Box, Relative Quantification Experiments



5 For Relative Quantification experiments: Select at least one **Reference** assay. In addition, specify the **PCR Efficiency (%)**, and if applicable, the **Error (%)**.

a Click **settings** .

b In the Analysis Settings dialog box, click the **RQ Settings** tab.

c Under **Reference Assay**, click the box in the row of the Assay you want as a reference.

d To specify the **PCR Efficiency (%)** and if applicable, the **Error (%)**, set the **RQ Algorithm** to **Efficiency Correction**.

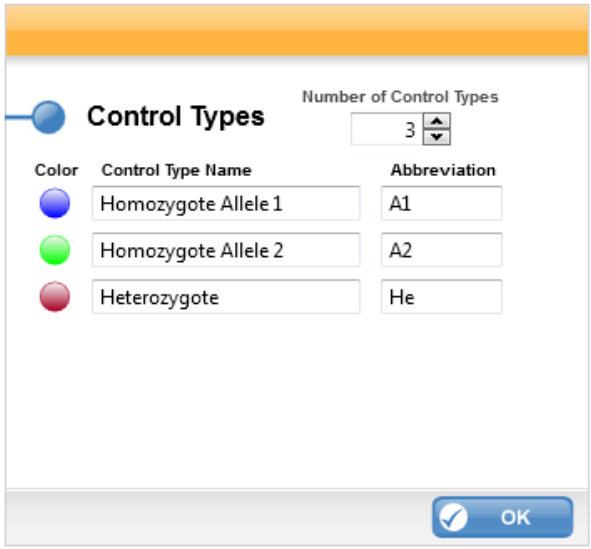
e Click **OK** .

NOTE
If the RQ Algorithm is set to ΔΔCq, you can only select one reference gene. In contrast, if the RQ Algorithm is set to Efficiency Correction, you can select up to three reference genes.

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.

Figure 4 Example of a Control Types Dialog Box for Genotyping



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. Click a new color on the color palette.

e Enter an **Abbreviation**.
f Click **OK** .

8 Proceed to set up samples.

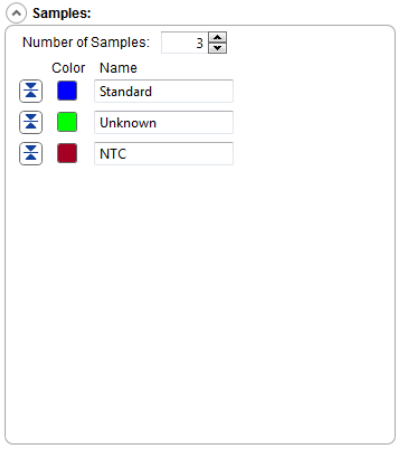
Set Up Samples

1 On the Plate Layout tab, click **Samples**.

2 Use the arrows to select the **Number of Samples**.

3 For each sample, enter a **Name** and select a **Color**.
To change the color, click the square color icon next to the sample name. Click a new color on the color palette.

Figure 5 Samples Section



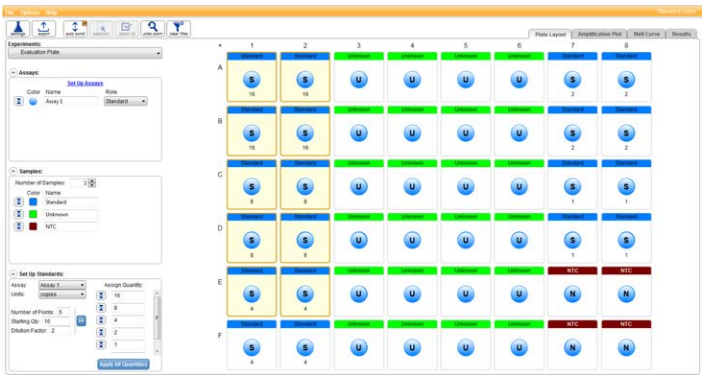
4 For Relative Quantification experiments: Select at least one **Reference** sample.
a Click **settings** .
b In the Analysis Settings dialog box, click the **RQ Settings** tab.
c Select the name of the **Reference Sample** in the drop-down list.
d Click **OK** .

5 For HRM experiments: Select at least one **Reference** sample.
a Click on a well.
b Right-click the well and click **Set Well as Reference**.
You can also choose a Reference from the Results tab.
a Click the **Results** tab.
b Select the name of the reference sample in the **Reference** drop-down list.

6 Proceed to assign assays and samples to wells.

Assign Assays and Samples to Wells

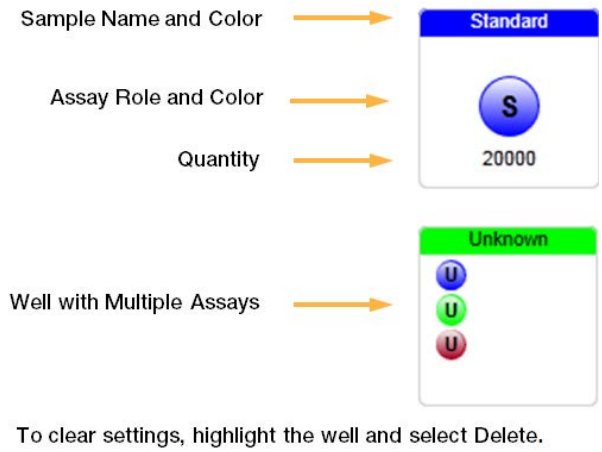
Figure 6 Plate Layout Tab, Assigning Assays and Samples



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 6.

2 Click the Apply / Unapply 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.

Figure 7 Well in the Plate Layout



3 To change the role of an assay in a given well, highlight the well and then select the desired **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.

Define Standards

When an Assay Role is set to Standard, define well standards in the Set Up Standards section on the Plate Layout tab.

1 Click one or more wells.

2 Select the **Assay**.

3 Select the **Units** to use.

4 Enter quantities in the **Assign Quantity** field.

Auto-Calculate Serial Dilutions

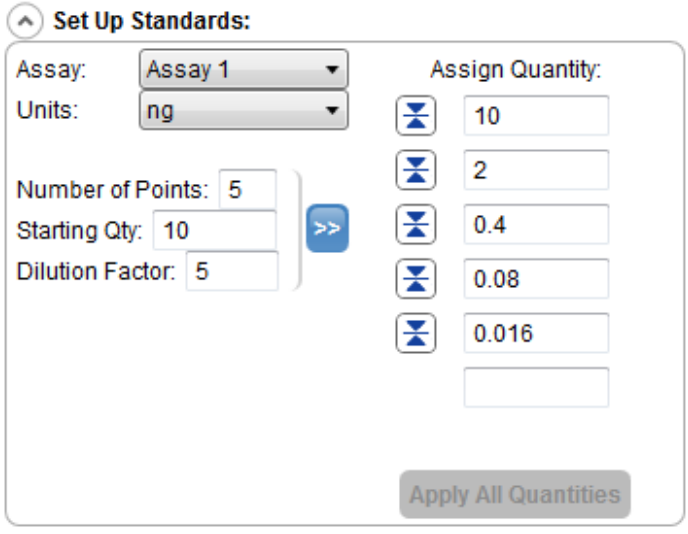
1 To auto-calculate serial dilutions:
   a Click one or more wells.
   b Enter the **Number of Points** in the standard curve.
   c Enter the quantity of the most concentrated standard in **Starting Qty**.
   d Enter the desired **Dilution Factor**.

2 Click the double-arrow >>.

3 Assign Quantity is now populated.

4 Click **Apply All Quantities**.

Figure 8 Serial Dilutions Auto-Calculated



Manually Enter Dilutions

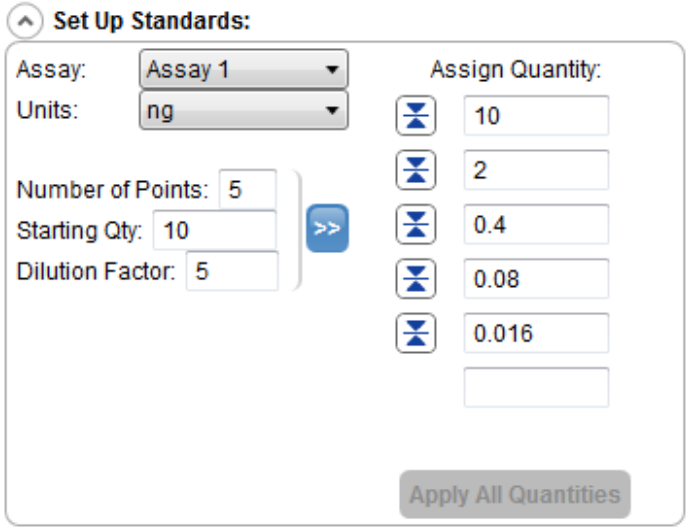
1 To manually enter dilutions:
   a Enter the **Number of Points** in the standard curve.
   b Enter the quantity of the most concentrated standard in **Starting Qty**.
   c Enter the desired **Dilution Factor**.

2 Click the double-arrow >>.

3 Assign Quantity is now populated.

4 In the Assign Quantity section, enter the actual number for each of the points on your standard curve.

Figure 9 Serial Dilutions Auto-Calculated



5 Click one or more wells.

6 Click the Apply / Unapply button next to the Assign Quantity number you want to assign to the wells.

Assign Standard Dilutions to Wells

You can assign standard dilutions to wells manually or automatically.

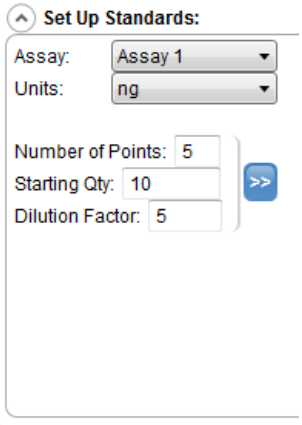
To assign dilutions automatically

1 Left-click and drag the mouse over a group of Standard Assay wells.
Make sure the assays in the wells selected are assigned the Role of Standard.

2 Click the **Apply / Unapply** button. Now all the wells you selected have the letter "S" on them.

3 In the Set Up Standards section, select the following:

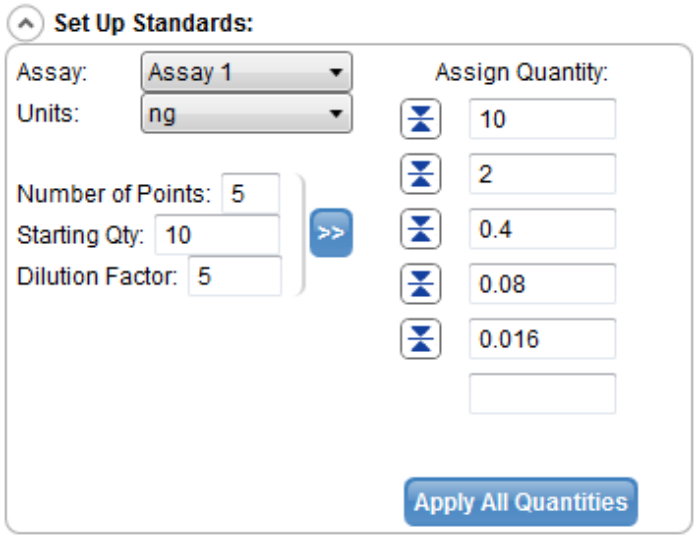
- **Assay**
- **Units**
- **Number of Points**
- **Standard Quantity**
- **Dilution Factor**



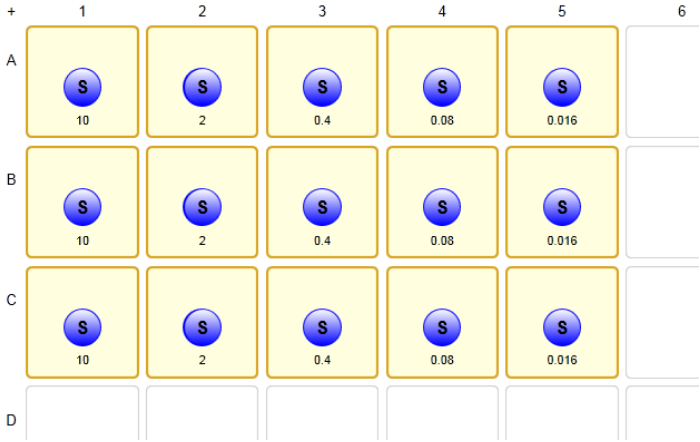
4 Click the >> double-arrow button to automatically assign the dilution quantities for your wells.

5 If EcoStudy recognizes your plate layout, it highlights the Apply All Quantities button.

6 Click **Apply All Quantities**.



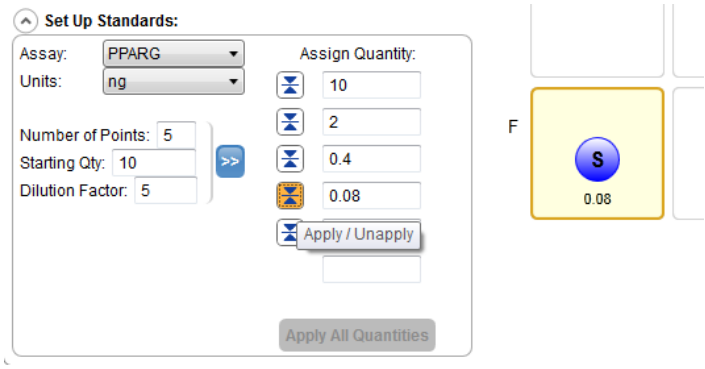
7 Now the wells show the dilution quantities on the plate layout.



To assign dilutions manually

Highlight one or more Standard Assay wells and click the **Apply / Unapply** button beside the appropriate dilution quantity (Figure 10).

Figure 10 Assigning Dilutions



Excluding, Including, and Clearing Wells

You can remove wells from analysis by excluding them. If you Exclude a well, the well keeps its related assay and sample information. Because the well retains its associated information, you can Include it again in your analysis.

You can Exclude or Include wells on the Plate Layout tab, the Amplification Plot tab, or the Results tab. Excluded wells display a large red X on the Plate Layout tab, and an E in a circle next to the well on the Amplification tab and the Results tab.

Clearing wells, in contrast, erases the assay and sample information of the well. It also excludes the well from analysis. You can Clear a well on the Plate Layout tab only.

NOTE

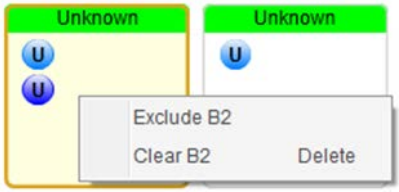
To Exclude or Clear a well, it must contain assay or sample information. To Include a well, it must be excluded first.

Exclude a Well

1 In the Plate Layout tab, select one or more wells you want to exclude from analysis.

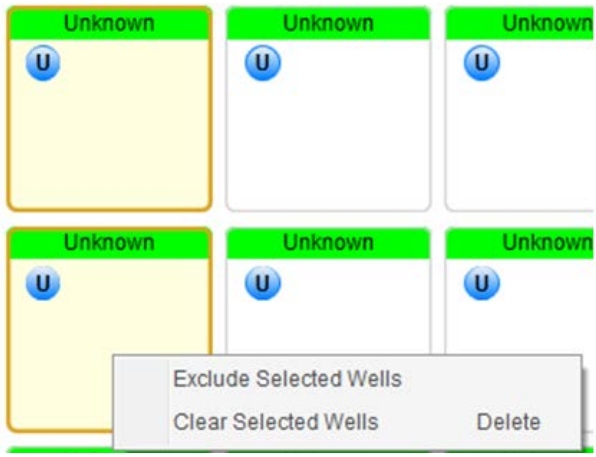
2 On the wells you select, right-click and click the **Exclude** option.
If you click one well to exclude, Exclude displays with the plate layout letter and number next to it.

Figure 11 Exclude or Clear One Well



If you select more than one well to exclude, **Exclude Selected Wells** displays.

Figure 12 Exclude or Clear Multiple Wells



Include a Well

1 In the Plate Layout tab, select one or more wells you excluded, but now want to include in the plate layout.

2 Right-click and click the **Include** option.
If you select one well to include, Include displays with the plate layout letter and number next to it.
If you select more than one well to include, Include Selected Wells displays.

Clear a Well

1 In the Plate Layout tab, select one or more wells you want to clear.

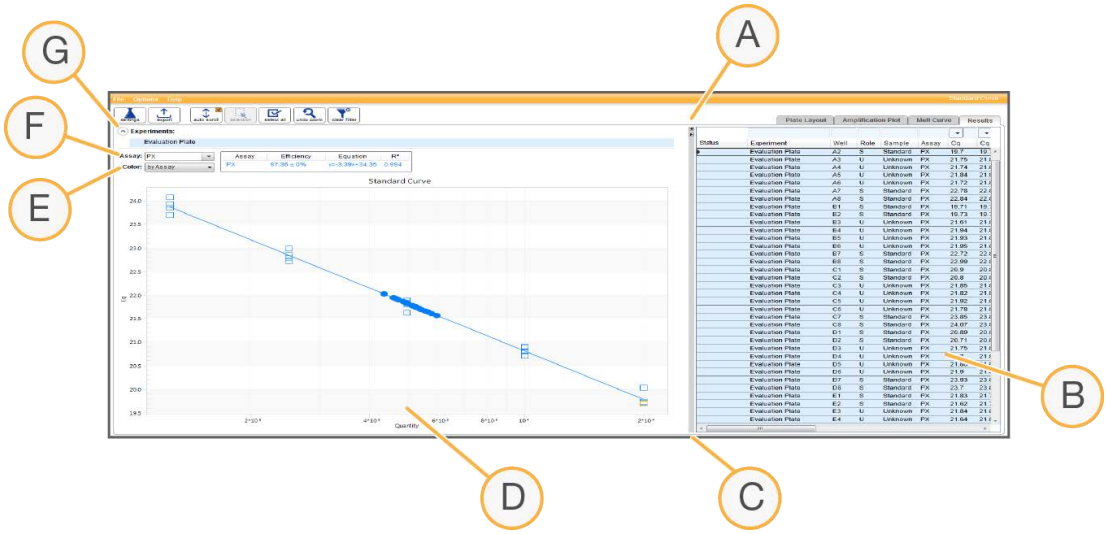
2 Press the Delete key on your keyboard. You can also right-click and click the **Clear** option.
If you select one well to clear, Clear displays with the plate layout letter and number next to it. See Figure 11 for an illustration.
If you select more than one well to clear, Clear Selected Wells displays. See Figure 12 for an illustration.

Analyzing Data

Chapter 3

Analyze Data

Figure 13 Results Tab for Analysis



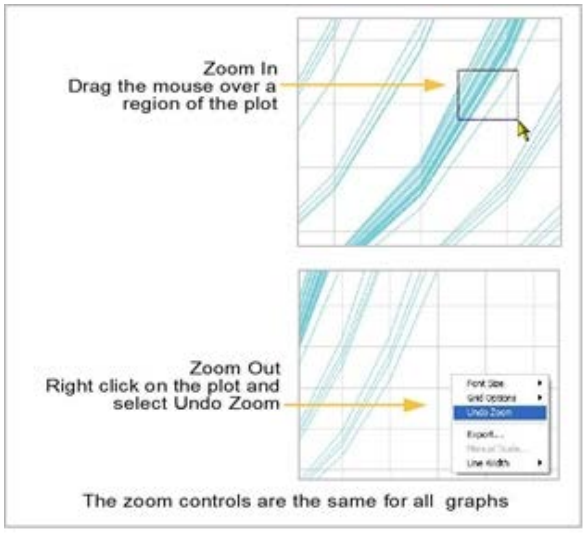
A Reset the display to show the graph or Well Table in full view, or return to split view
B Mouse over the rows in the Well Table to highlight the amplification curve highlighted in the graph
C Drag Vertical Bar to re-size panels
D Select the graph curves to highlight the corresponding row in the Well Table
E In the Color drop-down list, select by Assay or by Sample
F In the Assay drop-down list, select the identity of the assay
G Click on the arrow next to Experiments to see what experiments are included in your study

There are four tabs:

- Plate Layout
- Amplification Plot
- Melt Curve
- Results

The window controls are the same for each tab.

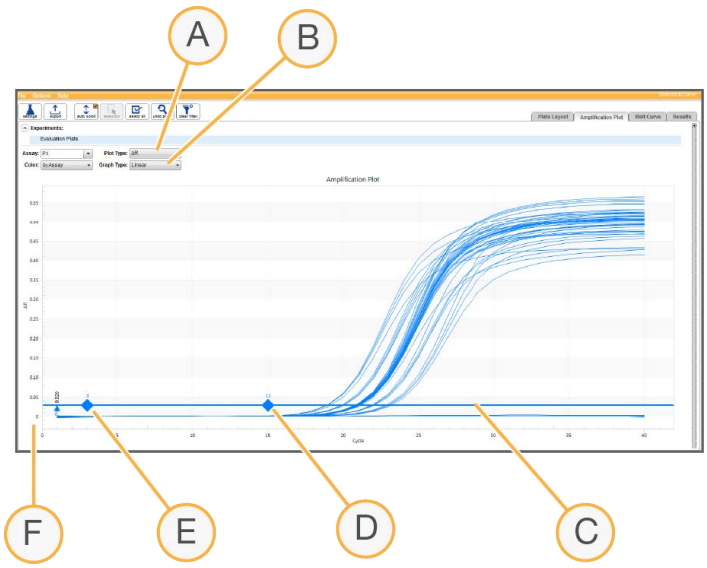
Figure 14 Zoom in and zoom out



Amplification Plot Tab

The Amplification Plot tab displays RFU versus the cycle number. You can toggle between 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 15 Amplification Plot Tab



A Toggle y-axis between Plot Type R, ΔR, Rn, and ΔRn
B Select Linear or Logarithmic scale graph types
C Threshold
D Baseline end
E Baseline start
F Linear scale

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.

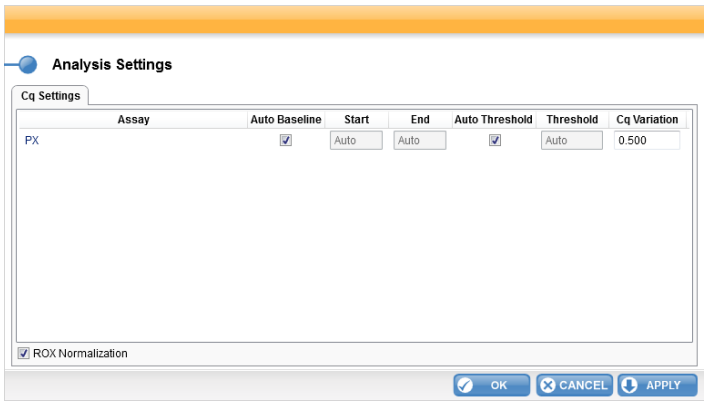
The Eco software uses a modification of the method of Liu and Saint (2002) to model the fluorescence plot as a line and a sigmoid. The model-fitting algorithm finds and subtracts the linear portion of the curve, and then the sigmoidal component is estimated.

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.

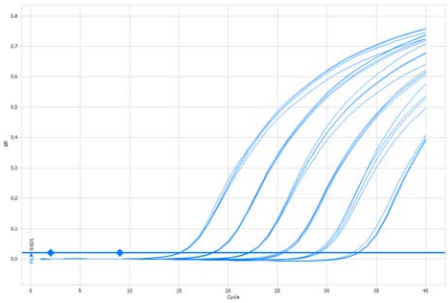
- To select the method of baseline determination, click **settings** or click **Options** | **Analysis Settings** from the main menu bar. The Analysis Settings box opens. Click the Auto Baseline check box to toggle between auto and manual baseline determination.

Figure 16 Analysis Settings Dialog Box for Auto Baseline



- 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 17 Baseline Adjustment



Threshold Adjustment

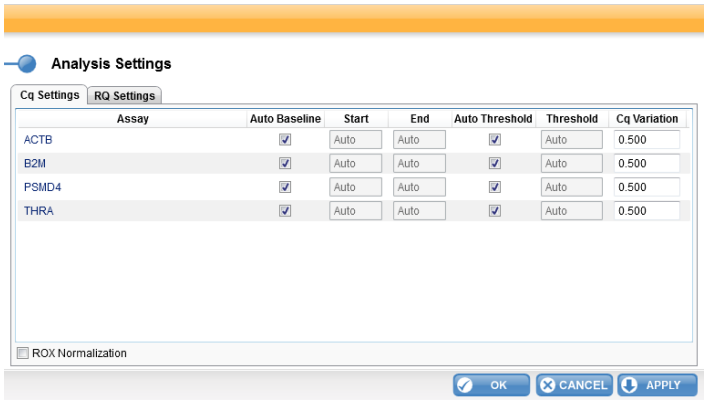
The Eco software uses a modification of the method of Liu and Saint (2002) to model the fluorescence plot as a line and a sigmoid. The model-fitting algorithm finds and subtracts the linear portion of the curve, and then the sigmoidal component is estimated.

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 the cycle 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.

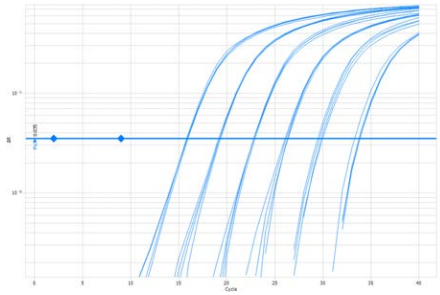
- To specify whether the threshold is determined automatically or manually, click **settings** or click **Options** | **Analysis Settings**. Analysis Settings opens. Click the **Auto Threshold** check box to toggle between auto and manual threshold determination.

Figure 18 Analysis Settings Dialog Box for Threshold Adjustment



- 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 log scale view, it should be set in the middle of the exponential phase, as shown in Figure 19.

Figure 19 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.

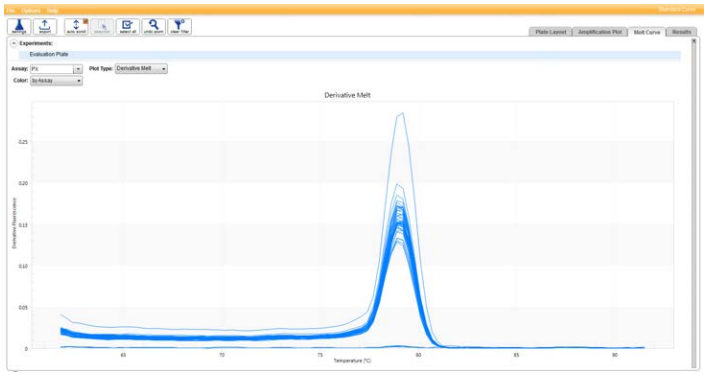
The Melt Curve tab displays the negative derivative of RFU versus temperature (-dRFU/dT) and calculates melting temperatures (Tm) based upon peak calls.

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 you only expect one amplicon. 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 20 Melt Curve Tab, Standard Melt

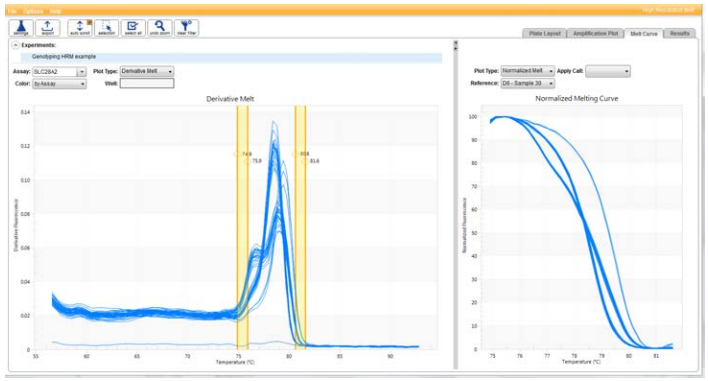


High Resolution Melt

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 or adjusted in the Analysis Settings dialog box.

Figure 21 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 list. 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 list 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.

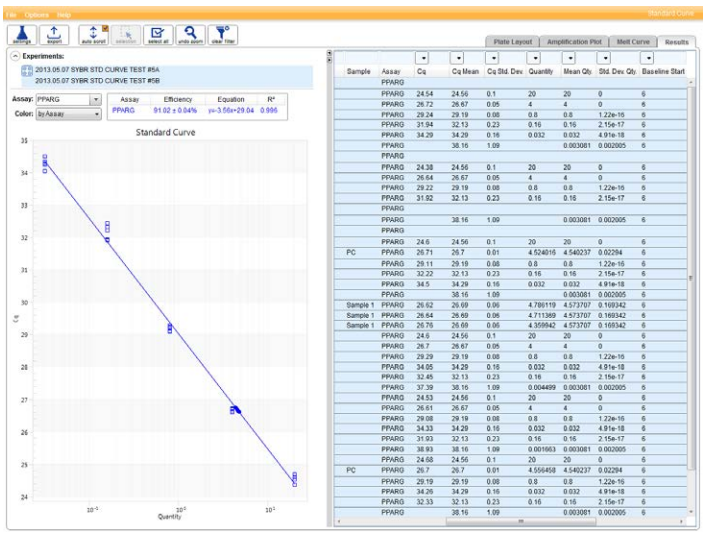
Results Tab

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 22). The slope, PCR efficiency, and R2 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** in the display. When a well is excluded from display, data is still calculated and included in analysis.

Figure 22 Results Tab, Example of a Standard Curve Display



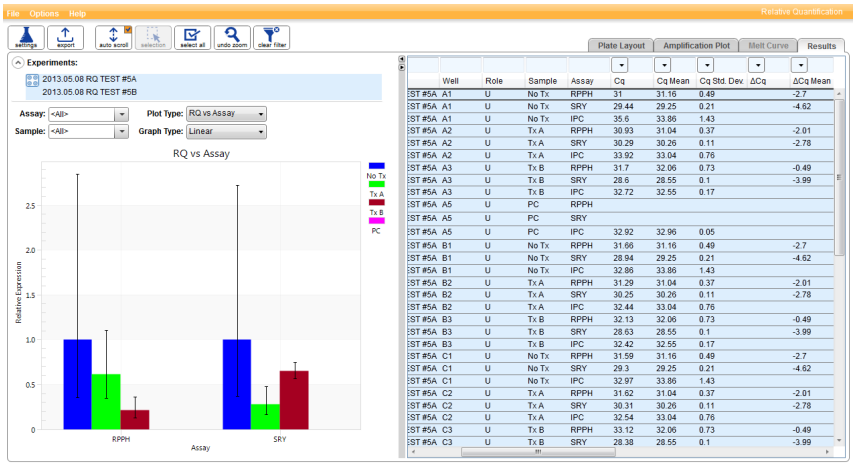
Relative Quantification

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 23 Results Tab, Example of a Relative Quantification 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.

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

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

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

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

Standard Mean and Standard Deviation

For each of the assays at each of the wells, Eco computes an amplification trace from which it derives a Cq value. The Cq value is the last cycle number at which the threshold intersects with the amplification trace. Using the following calculation, Eco determines the Cq mean and Cq standard deviation for all the traces from the same assay in a technical replicate group.

$$Cq_{mean} = \frac{1}{n}\sum_{i=1}^{n} Cq_i$$

$$S_{cq} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(Cq_i - Cq_{mean})^2}$$

Standard Variation Value

For each daughter plate, Eco computes a Cq standard variation value. The standard variation value is calculated by subtracting the average of the daughter's plate control Cq value from the corresponding quantity in the mother plate.

$$Cq\ Variation = \left| \frac{1}{n} \sum_{i=PC}^{mother\ plate} Cq_i - \frac{1}{m} \sum_{j=PC}^{daughter\ plate} Cq_j \right|$$

Standard Error

Eco uses the calculation below to determine standard error in a Relative Quantification study. In this formula, S(E) is equal to the standard error of the efficiency.

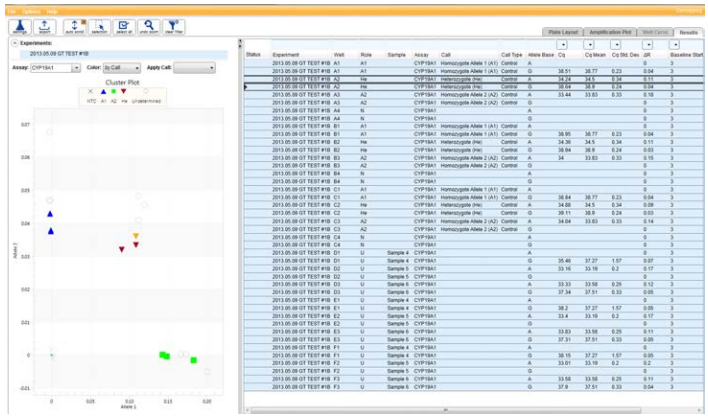
$$S(RQ^{Sample}_{TargetAssay}) = \sqrt{RQ^{Sample}_{TargetAssay}\text{^}2 \times \left[\left(\frac{\Delta \overline{Cq}^{TargetSample}_{Assay} S(E)}{E} \right)^2 + \left(\ln(E)\, S^{TargetSample}_{Assay} \right)^2 \right]}$$

Genotyping

Genotyping (allelic discrimination) experiments generate a scatter plot (Figure 24). Bi-allelic genotyping results in three potential clusters of end-point fluorescence data. Best results show each allele group distinct from the others. Allele names are user-defined within the Control Types dialog box, available from the **Options** menu at the top of the screen.

NOTE
Make sure to use the scroll bar at the bottom of the well table to see all sorting options.

Figure 24 Results Tab, Example of an Allelic Discrimination Display



Grey circles indicate that an allele type has not been called. Click the **selection** icon to apply a call. 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
No analysis settings are available for a single-read genotyping experiment.

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 to maximize cluster resolution. The Difference Plot displays melt curves relative to the sample in the well selected from the Reference drop-down list at the top of the graph. Use the Plot Type drop-down list to toggle between Normalized Melt and Difference Melt.

Figure 25 Results Tab, Example HRM display of Normalized Melt Curve

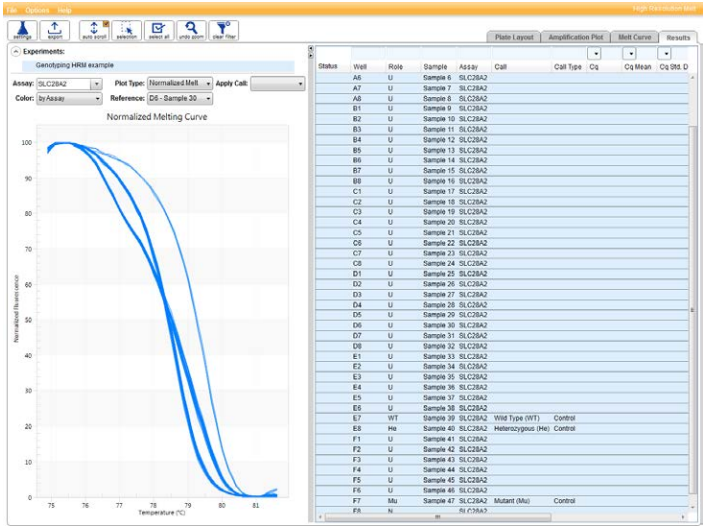
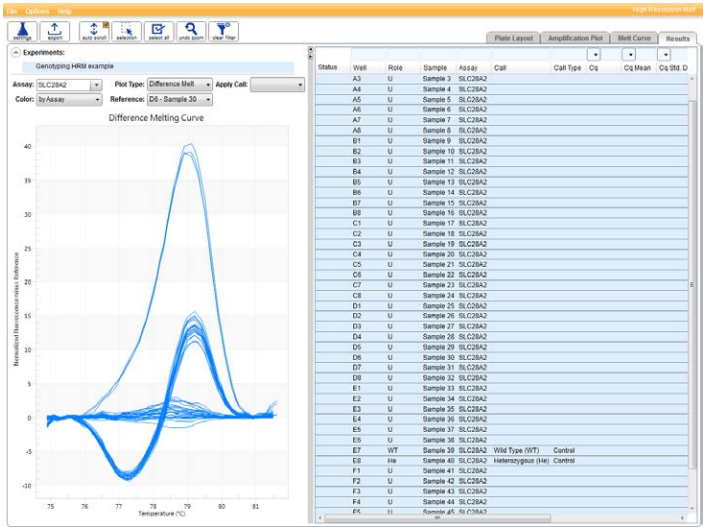
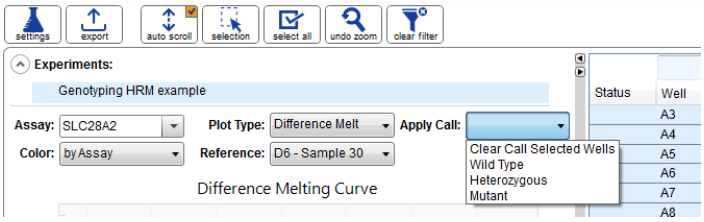


Figure 26 Results Tab, Example HRM display of Difference Melting Curve



You can manually assign a call to a given sample by clicking **selection** and drawing a line across the melt curve. Then, in the **Apply Call** box, select the appropriate genotype.

Figure 27 Results Tab, Apply Call Box



Setting Up a Study

Chapter 4

Setting Up a Study

The process to set up a study is outlined here, and is described in the following sections of this document.

1 Open the EcoStudy software and add one or more experiments to a study. If adding more than one experiment:
- The experiments must be of the same type; Standard Curve, Relative Quantification, or Genotyping
- The experiments must have the same thermal profile (except for melt profile)
- The experiments must have the same number of thermal cycles

Steps two and three are needed for Standard Curve or Relative Quantification studies with more than one experiment:

2 Select one experiment as the mother plate. The mother plate is the plate against which the other experiments in the study will be compared. EcoStudy automatically selects one experiment to serve as the mother plate. However, you can change this setting to designate any other experiment as the mother plate.

3 Select at least one well on the mother plate to serve as a plate control. When assigned on the mother plate, EcoStudy automatically identifies wells containing the same assays, reporter dyes, and sample name on the other plates in your study. During analysis, the EcoStudy software uses the Cq data from the plate control wells identified on each plate as a basis for comparing values across plates.

Adding an Experiment

To analyze data from multiple experiments in a study, you must compare two or more experiments of the same type (Standard Curve, Relative Quantification or Genotyping). The experiments must also have the same thermal profile and number of thermal cycles. The melt profile does not have to be the same.

1 Use the startup screen options to open an experiment or study.

2 Click **File** | **Add Experiment**.

TIP
You can also add experiments by dragging-and-dropping experiments onto the EcoStudy software or by right-clicking the Experiment list on the Amplification Plot, Melt Curve, or Results tab and clicking **Add Experiment** in the context menu.

3 Navigate to the *.ecod experiment data files you want to include in your study and click **Open**.

TIP
If the experiment files are saved in the same folder, you can Ctrl- or Shift-click to select multiple files for inclusion in your study at once.

4 Repeat this procedure until all desired experiments are added to your study.

NOTE
EcoStudy automatically selects one experiment to serve as the *mother plate* in a Standard Curve or Relative Quantification study. The mother plate is the plate against which the other experiments in the study will be compared. You can change this setting to designate any other experiment as the mother plate. For more information, see *Designating the Mother Plate* on page 41.

NOTE
If one or more of the experiments you selected is of a different type, has a different thermal profile, or has a different number of thermal cycles than the first experiment added to the study, an error message opens for each unsupported experiment. Click **OK** through each error message. EcoStudy will add any remaining supported experiments to your study.

Requirements for a Standard Curve Study

To ensure successful analysis, a Standard Curve study must contain:

- One or more experiments of the same type, with the same thermal profile (except for melt profile) and number of thermal cycles
- One mother plate (if the study includes more than one experiment)
- At least two standards with different values
- One or more plate control wells in each plate

Requirements for a Relative Quantification Study

To ensure successful analysis, a Relative Quantification study must contain:

- One or more experiments of the same type, with the same thermal profile (except for melt profile) and number of thermal cycles
- One mother plate (if the study includes more than one experiment)
- One or more plate control wells in each plate
- One reference sample against which the target (non-reference) samples will be analyzed
- At least one target (non-reference) assay

Requirements for a Genotyping Study

To ensure successful analysis, a Genotyping (allelic discrimination) study must contain:

- One or more experiments of the same type, with the same thermal profile (except for melt profile) and number of thermal cycles
- One or more plate control wells in each plate

Requirements for an HRM Study

To ensure successful analysis, an HRM study must contain:

- One reference sample against which the target (non-reference) samples will be analyzed
- One or more plate control wells in each plate

NOTE
HRM (High Resolution Melt) experiments cannot be combined for analysis in EcoStudy.

Designating the Mother Plate

Every Standard Curve or Relative Quantification study must have one experiment designated as the *mother plate*. The mother plate is the plate against which the other experiments in the study will be compared. The other experiments in the study are referred to as *daughter plates*. If yours is not a Standard Curve or Relative Quantification study, go on to *Introduction* on page 44.

1. Click the **Plate Layout** tab.
2. From the Experiments drop-down list, select the experiment you want to use as the mother plate for your study.
   - For Standard Curve studies, the mother plate must have at least two wells with the role Standard, but with different quantities.
   - For Relative Quantification studies, any plate can be used as the mother plate. However, the mother plate must have at least one well that meets the criteria to serve as a plate control. For more information on plate control requirements, see *Designating Plate Control Wells in your Study* on page 42.
3. Click the **Mother Plate** check box.
   The previously designated mother plate is demoted to be a daughter plate.

NOTE
If you had a plate control well designated on the previous mother plate, it is cleared and a new plate control will need to be assigned on the new mother plate. For more information, see *Designating Plate Control Wells in your Study* on page 42.

Designating Plate Control Wells in your Study

For a Standard Curve or Relative Quantification study, at least one well on the mother plate must be designated as the *plate control*. Once assigned on the mother plate, EcoStudy automatically identifies wells containing the same assays, reporter dyes, and sample name on the other plates in your study. During analysis, the EcoStudy software uses the Cq data from the plate control wells identified on each plate as a basis for comparing values across plates. If yours is not a Standard Curve or Relative Quantification study, go on to *Introduction* on page 44.

1 Click the **Plate Layout** tab.

2 From the Experiments drop-down list, select the experiment designated as the mother plate for your study.

3 Right-click the desired well on the plate layout diagram and select **Assign as Plate Control**.

- For Standard Curve studies, the well you designate as the plate control must have an assay with the role **Standard**, **Unknown**, or **Positive**, as well as have a sample assigned. Additionally, the plate control well must not be excluded from the study.
- For Relative Quantification studies, the well you designate as the plate control must have an assay with the role **Unknown** or **Positive**, as well as have a sample assigned. Additionally, the plate control well must not be excluded from the study.

Wells containing the same assays, reporter dyes, sample name, and assay names are labeled **Plate Control** on the plate layout diagram for every experiment in your study. On the other tabs in the EcoStudy software, plate control wells are indicated by the C icon in the Well Table.

Specifying Analysis Settings

Chapter 5

Introduction

As you change settings in your study, including adding experiments and changing the mother plate and plate control designations, the EcoStudy software automatically re-analyzes your data using the default baseline, threshold, and Cq variation values. If you want to use values other than the default settings, you can specify so in the Analysis Settings dialog box, as described in the following sections.

Analyzing a Standard Curve Study

1 Click **settings**  .

2 On the Cq Settings tab, for each assay, do the following:

a Determine whether you want to specify the baseline start and end values or allow the EcoStudy software to auto-calculate those values.

If you specify the baseline Start and End values yourself, you can use any numeric value between 1 and the total number of cycles in your study. The baseline End value must be equal to or higher than the baseline Start value.

b Determine whether you want to specify the threshold or allow the EcoStudy software to auto-calculate the value.

If you specify the Threshold value yourself, you can use any numeric value above 0 (zero).

NOTE

For more information on the auto-baseline and auto-threshold algorithms, see the *Eco Real-Time PCR System User Guide*.

c Specify the desired Cq Variation value.

If a plate control well in your study has a Cq Variation value above the one you specify, it is indicated by a  icon in the Well Table, so you can exclude that well from the analysis, if desired. To exclude the well, right-click it in the Well Table and click **Exclude**.

3 Click **OK**.

EcoStudy re-analyzes your data using the new analysis settings.

Analyzing a Relative Quantification Study

1 Click **settings**  .

2 On the RQ Settings tab, do the following:

a From the **RQ Analysis Method** drop-down list, specify the desired analysis method: **Singleplex** or **Multiplex**.

— If your study includes only singleplexed wells, the drop-down list only includes that one option.

— If your study includes both singleplexed and multiplexed wells, both options are available. Only the wells matching your selection will be analyzed. The other wells will be omitted from the analysis.

b From the **RQ Algorithm** drop-down list, specify the desired normalization method: **ΔΔCq** or **Efficiency Correction**.

The ΔΔCq method is supported only if you have a single reference gene and the amplification efficiencies are equal and at 100%. If your study does not meet these requirements, you must use the Efficiency Correction method. For more information on these methods, see the *Eco Real-Time PCR System User Guide*.

c From the **Reference Sample** drop-down list, select one sample to be used as the reference sample.

d For each assay, specify:

— **PCR Efficiency %**

— **Error %**: can be any numeric value greater than or equal to 0 (zero)

e Select the **Reference Assay** check box for every assay you want to use as a reference assay.

You must leave at least one assay unchecked to be used as the target assay.

3 On the Cq Settings tab, for each assay, do the following:

a Determine whether you want to specify the baseline start and end values or allow the EcoStudy software to auto-calculate those values.

If you specify the baseline Start and End values yourself, you can use any numeric value between 1 and the total number of cycles in your study. The baseline End value must be equal to or higher than the baseline Start value.

b Determine whether you want to specify the threshold or allow the EcoStudy software to auto-calculate the value.

If you specify the Threshold value yourself, you can use any numeric value above 0 (zero).

NOTE

For more information on the auto-baseline and auto-threshold algorithms, see the *Eco Real-Time PCR System User Guide*.

c Specify the desired Cq Variation value.

If a plate control well in your study has a Cq Variation value above the one you specify, it is indicated by a  icon in the Well Table, so you can exclude that well from the analysis, if desired. To exclude the well, right-click it in the Well Table and click **Exclude**.

4 Click **OK**.

EcoStudy re-analyzes your data using the new analysis settings.

Analyzing a Genotyping Study

NOTE
Analysis settings are not available for single-read genotyping experiments.

1 Click **settings** .

2 On the Cq Settings tab, for each assay, do the following:

a Determine whether you want to specify the baseline start and end values or allow the EcoStudy software to auto-calculate those values.

If you specify the baseline Start and End values yourself, you can use any numeric value between 1 and the total number of cycles in your study. The baseline End value must be equal to or higher than the baseline Start value.

b Determine whether you want to specify the threshold or allow the EcoStudy software to auto-calculate the value.

If you specify the Threshold value yourself, you can use any numeric value above 0 (zero).

NOTE
For more information on the auto-baseline and auto-threshold algorithms, see the *Eco Real-Time PCR System User Guide*.

3 Click **OK**.

EcoStudy re-analyzes your data using the new analysis settings.

NOTE
The Melt Curve tab is not available because a melt curve stage is not used in genotyping experiments.

Analyzing a High Resolution Melt Study

When analyzing an HRM study, you will set both HRM and Cq settings if your experiment on Eco included amplification. If your experiment on Eco did not include amplification, select HRM settings only.

HRM Settings

The HRM dialog box sets four temperatures for analysis. The temperature settings are, in order, Pre-Melt Start, Pre-Melt End, Post-Melt Start, and Post-Melt End.

The temperature settings can be specified directly on the Derivative Melt plot in the Melt Curve tab. Slide each of the four vertical orange lines to the temperature you want. The Analysis Settings dialog box will automatically populate with your selection. See *High Resolution Melt* on page 28.

1. Click **settings** .
2. Click the **HRM Settings** tab.
3. Enter the **Pre-Melt Start** temperature. This temperature is the beginning point, before the melt region of the curve, when DNA still has two strands and has maximum fluorescence.
4. Enter the **Pre-Melt End** temperature. This temperature is the end point, before the melt region of the curve, when DNA still has two strands and has maximum fluorescence. Pre-Melt End is immediately before the curve begins to contract the most vertically, right before the DNA starts to melt.
5. Enter the **Post-Melt Start** temperature. This temperature is the point when DNA strands have completely separated, after the melt region curve, and when there is no fluorescence.
6. Enter the **Post-Melt End** temperature. This temperature is the end point of the region of the curve when DNA strands have completely separated and there is no fluorescence. The curve is completely flat.
7. **Set to Default** places the Pre-Melt and Post-Melt temperature values where the software detects a minimum difference between the change of fluorescence values between conditions before and after the main melt event.
8. If your experiment on Eco included amplification, click the **Cq Settings** tab. If not, click **OK**. EcoStudy re-analyzes your data using the new analysis settings.

Cq Settings

NOTE
The Cq Settings tab is available if your HRM experiment contains a PCR stage..

1. Click **settings** .
2. On the Cq Settings tab, for each assay, do the following:
   a. Determine whether you want to specify the baseline start and end values or allow the EcoStudy software to auto-calculate those values.
   If you specify the baseline Start and End values yourself, you can use any numeric value between 1 and the total number of cycles in your study. The baseline End value must be equal to or higher than the baseline Start value.

b Determine whether you want to specify the threshold or allow the EcoStudy software to auto-calculate the value.
If you specify the Threshold value yourself, you can use any numeric value above 0 (zero).

NOTE
For more information on the auto-baseline and auto-threshold algorithms, see the *Eco Real-Time PCR System User Guide*.

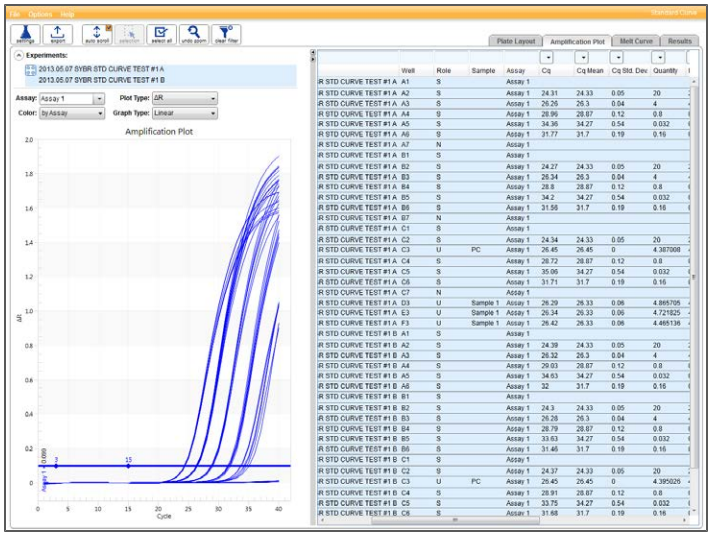
3 Click **OK**.
EcoStudy re-analyzes your data using the new analysis settings.

Reviewing Data in a Study

Chapter 6

Introduction

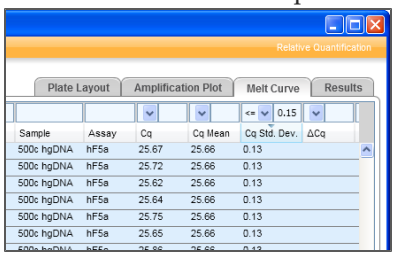
The data in your study can be viewed graphically or in a table on the Amplification Plot, Melt Curve, and Results tabs in the EcoStudy software. The example here shows the Amplification Plot tab for a Relative Quantification study containing three experiments.



Well Table

The Well Table appears on the Amplification Plot, Melt Curve, and Results tabs. The columns in the Well Table vary depending on your type of study. Use the Well Table settings to control what data are included in the Amplification Plot, Melt Curve, and Results graphs.

- Select specific rows in the Well Table to display the data in the graph.
  By default, all rows are selected (highlighted in yellow) and so data for all wells in your study are shown in the graph.
- Hover over a row in the Well Table to highlight it in the graph.
- Specify filter criteria in the empty fields above each column to find data more easily.
  In this example, the Well Table is filtered to show only wells with a Cq Standard Deviation less than or equal to 0.15.



  To clear the filter and return to the full data set, click **clear filter** .
- Select a column heading to sort the Well Table by the data in that column.
- Click the **Disable Auto Scroll** button to stop the Well Table from scrolling vertically as you highlight data in the graph.

Exclude a Well

1. In the well table, click one or more wells you want to exclude from analysis.
2. Right-click and click **Exclude**.

An Exclude Symbol appears in the Status column next to the excluded well.

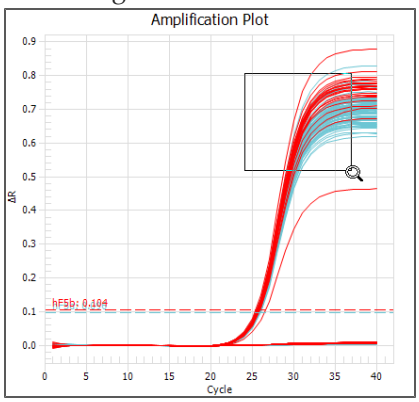
Include a Well

1. In the well table, click one or more wells you excluded, but now want to include in your analysis.
2. Right-click and click **Include**.

Amplification Plot

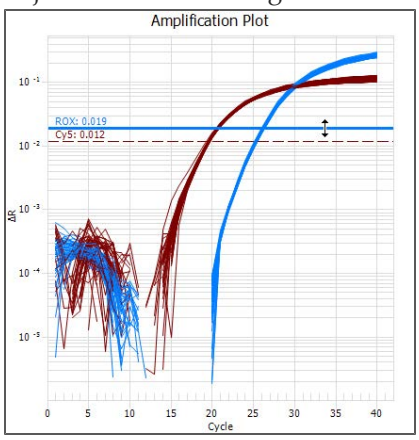
The Amplification Plot displays the normalized or baseline-subtracted fluorescence signal for a well at each cycle. Using the Amplification Plot settings, you can control how data are displayed in the Well Table and graph.

- Hover over a line in the Amplification Plot to highlight it in the Well Table.
- From the **Experiments** list, select one or more experiments to limit what data appear in the graph and Well Table.
- From the **Assay** drop-down list, select one or more assays to limit what data appear in the graph and Well Table.
- From the **Plot Type** drop-down list, specify whether you want to show normalized (R) or baseline-subtracted (ΔR) data in the graph.
- From the **Graph Type** drop-down list, specify whether you want to show the data using a logarithmic or linear scale.
- Click-drag the mouse over an area of the graph to zoom in on that area.



To clear the zoom and return to the full view of the graph, click **undo zoom** .

- Drag the threshold line for an assay up or down on the graph to change the analysis settings without opening the Analysis Settings dialog box. The threshold can only be adjusted when viewing the data on a log scale.

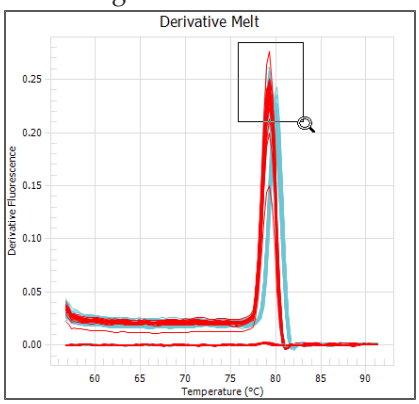


- If you turned off the Auto Baseline option in the Analysis Settings dialog box, you can drag the baseline start and end point ◆ indicators for an assay left or right on the graph to change the baseline settings.

Melt Curve

The Melt Curve graph displays the fluorescence signal or derivative fluorescence signal for a well at every 0.3° C (0.54° F) of melting. Using the Melt Curve settings, you can control how data are displayed in the Well Table and graph.

- Hover over a line in the Melt Curve graph to highlight it in the Well Table.
- From the **Experiments** list, select one or more experiments to limit what data appear in the graph and Well Table.
- From the **Assay** drop-down list, select one or more assays to limit what data appear in the graph and Well Table.
- From the **Plot Type** drop-down list, specify whether you want to show Raw or Derivative Melt data in th graph.
- Click-drag the mouse over an area of the graph to zoom in on that area.



To clear the zoom and return to the full view of the graph, click **undo zoom** .

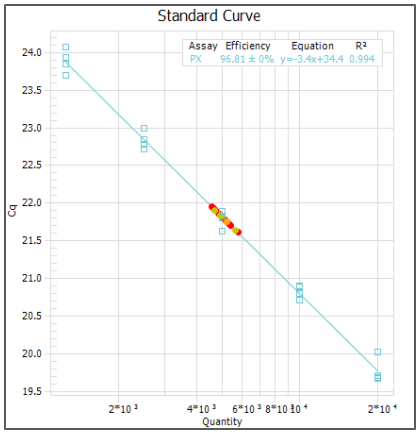
Results Tab

The Results graph displays the results of your study in one of the following formats, depending on the type of study you ran: Standard Curve, Relative Quantification, Genotyping, or HRM.

NOTE
The algorithm for auto-baseline and auto-threshold is improved in EcoStudy v5. As a result, if you compare the data of previous versions to v5 data, you may see small differences in the absolute Cq values.

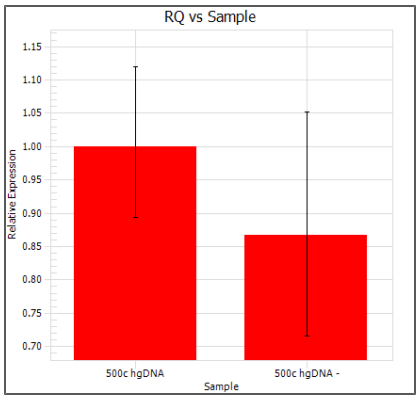
Standard Curve

Standard Curve studies generate a standard curve line graph. The slope, PCR efficiency and standard error percentage, and R^2 of that curve appear in the graph's legend. The standard curve is always shown, though you can decide which data points to include in the graph by selecting the appropriate rows in the Well Table.



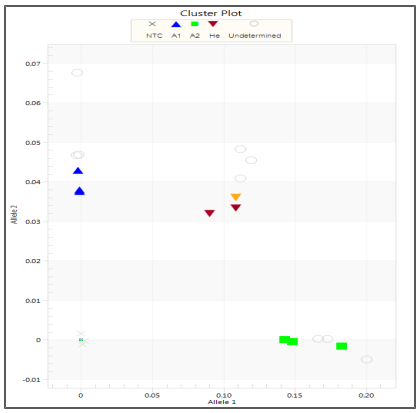
Relative Quantification

Relative Quantification studies generate a bar graph that includes error bars. The displayed histogram can be toggled to group data by assay or by sample.



Genotyping

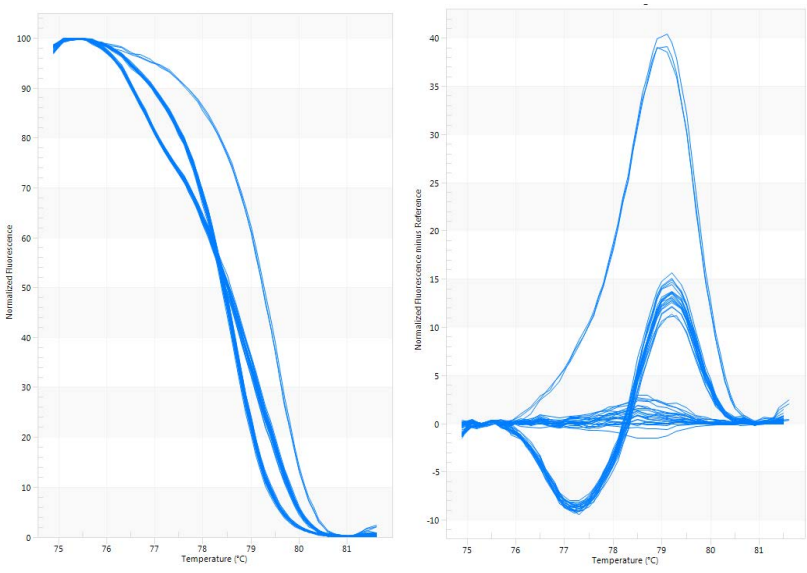
Genotyping studies generate a cluster plot. The different colors on the cluster represent different samples. Grey circles indicate allele types that are not called.



HRM

Only one HRM experiment at a time can be made into a study. HRM studies generate a normalized melt curve and a difference melting curve. Toggle between Normalized Melt and Difference Melt to see each plot type.

Figure 28 Normalized Melt (Left) and Difference Melt (Right)



Working with Templates and Sample Sheets

Chapter 7

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.	

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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.

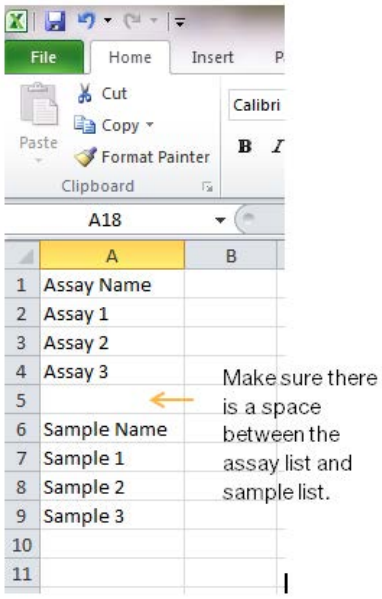
3 In the same column, enter the heading “Assay Name” and “Sample Name”.

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 29 Example of a Sample Sheet



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.
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.

Term	Definition
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.

Term	Definition
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).

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

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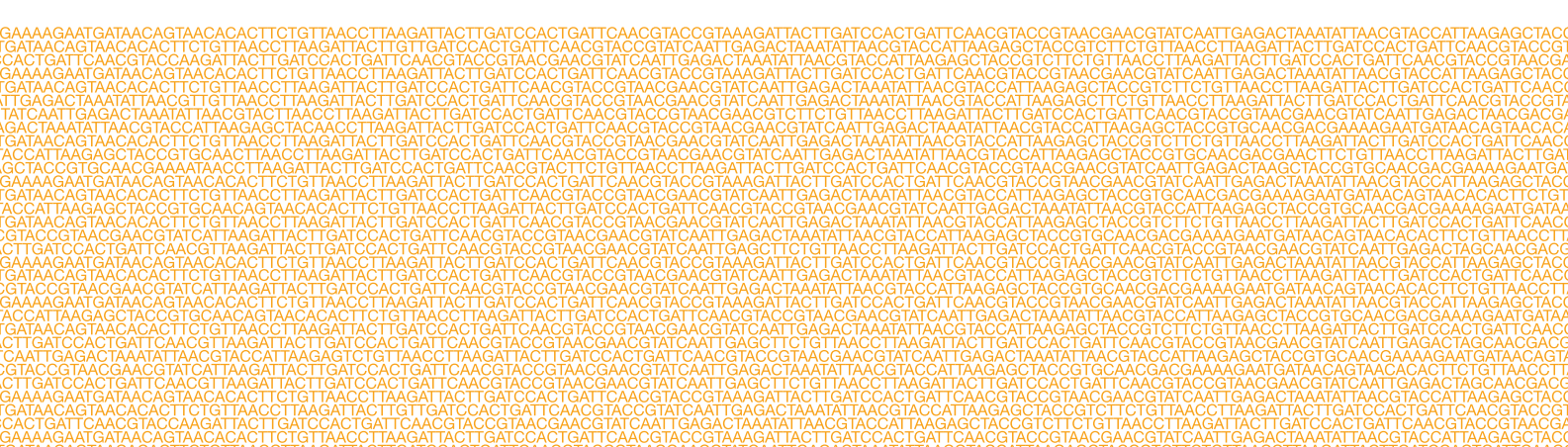

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Term	Definition
Standard Deviation (SD)	The SD of replicate Cq measurements is a measure of the precision of the assay.
TAMRA	Tetramethyl-6-carboxyrhodamine. Commonly used as a quencher.
Target	The DNA or RNA sequence to be amplified.
Template	See 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).

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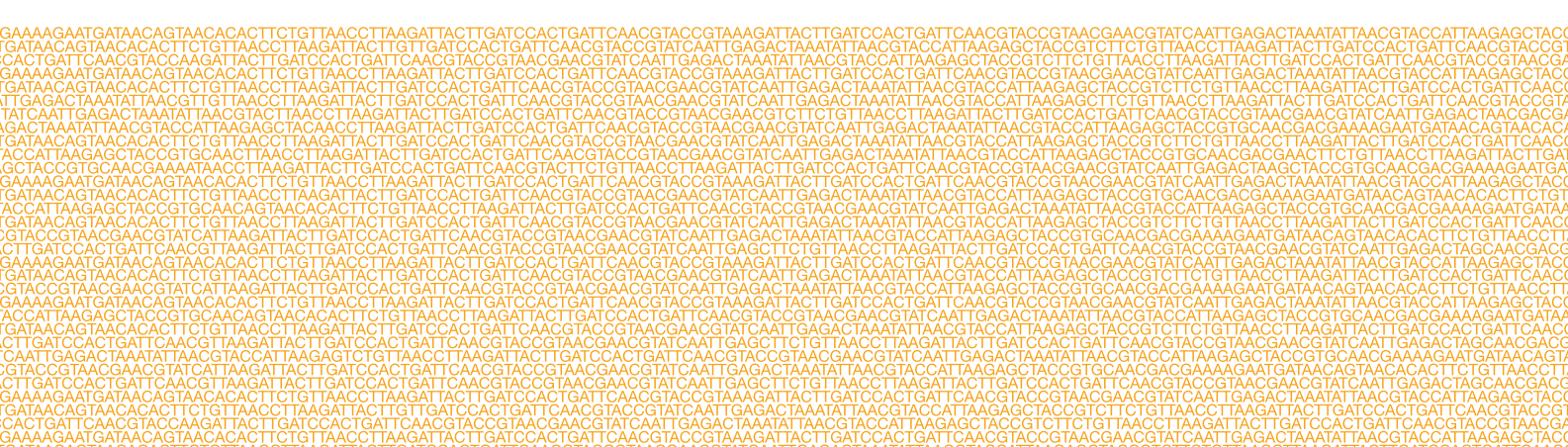

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