Eco™ Thermal and Optical Systems Deliver High Precision

Gage R&R study of the Eco Real-Time PCR System demonstrates high well-to-well, plate-to-plate, and system-to-system uniformity that supports high-performance applications and reduces the need for multiple replicates per run.

Introduction

By detecting and quantitatively monitoring amplified DNA as PCR progresses, real-time PCR (quantitative or qPCR) instruments provide a robust approach for gene expression, genotyping, copy number variation, mutation screening, and methylation analysis. Data quality and reproducibility are governed by how robust a real-time PCR instrument's thermal and optical systems are, with precise thermal cycling and sensitive optical detection critical for high-performance applications such as high resolution melt (HRM) curve analysis.

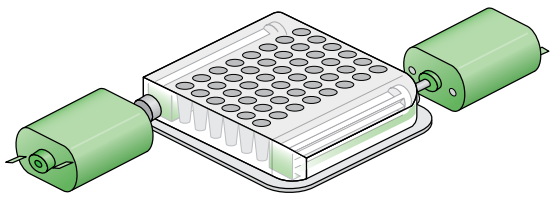
The thermal and optical units of the Illumina Eco Real-Time PCR System deliver thermal stability with ± 0.1°C well-to-well uniformity, and sensitive fluorescence detection down to a single copy. To evaluate the impact of these units on the precision of the Eco instrument, a Gage reliability and reproducibility (Gage R&R) study was performed. Two different operators performed the study, running the same assay in triplicate using four unique fluorescent reporters, on four different Eco systems.

Proprietary Thermal System

Real-time PCR specificity and efficiency depend upon precise temperature control during denaturation, annealing, and extension steps. For the highest accuracy, a real-time PCR thermal system must maintain a uniform temperature across the entire heat block, ensuring that all samples proceed through the reaction equally. In traditional real-time PCR instruments, thermal cycling is performed by Peltier-heated solid metal thermal blocks. Heating a solid metal block to the required temperature takes significant energy and time, with most systems designed to overshoot the desired temperature by several degrees before equilibrating at a plateau temperature, creating a substantial lag time to allow all wells to reach the desired temperature. The lack of uniform heating throughout the thermal block contributes to thermal non-uniformity (TNU) values of ±0.5°C, poor thermal ramp rates, and long run times, impacting the suitability of these systems for high-performance applications.

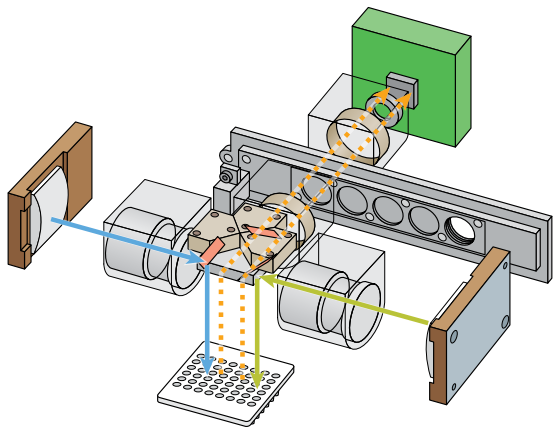
In contrast, the Eco thermal system incorporates a precisely electro-formed hollow silver block containing conductive fluid, which is heated and cooled by a single Peltier device (Figure 1). During PCR cycling, opposing agitators rapidly circulate the fluid throughout the hollow block, transferring heat from the Peltier device evenly across all wells. This minimizes the amount of temperature overshoot required at each plateau, reducing lag time and leading to faster runs that more closely follow the user-defined thermal profile. The Eco system's unique design eliminates edge effects (outer wells at a lower temperature than inner wells) and results in robust thermal performance, with TNU values below 0.1°C, thermal ramp rates of 5.5°C/sec, and reduced run times of < 40 min for 40-cycle PCR protocols.

Figure 1: Eco Thermal System



The Eco thermal system consists of a precisely electroformed 48-well hollow silver block containing conductive fluid. The block is heated and cooled by a single Peltier device, with an agitator assembly consisting of two paddles driven by electromagnetic motors. During PCR cycling, the paddles move, rapidly circulating the fluid across the 48 wells, allowing the block to achieve high ramp rates and low TNU, thus reducing the overall experiment time.

Figure 2: Eco Optical System



The Eco optical system consists of two 48-fixed LED panels that provide fluorescent dye excitation over a broad spectrum, with each of the 48 wells individually illuminated to minimize cross-talk between wells. Four emission filters in a linear filter slide and a high-performance CCD camera detect the fluorescence from each sample target at each cycle.

High-Performance Optical System

Real-time PCR uses fluorescent reporters to detect amplification of nucleic acid targets after each PCR cycle. The fluorescence signal is captured in real time to enable quantitative analysis at the appropriate phase of the reaction. The Eco system facilitates four-color multiplex applications and is calibrated for use with SYBR, FAM, HEX, VIC, ROX, and Cy5 dyes, but can be used with any real-time PCR chemistry. Its optical system uses light emitting diodes (LEDs), which are very stable over their lifetime, contributing to accurate data generation and increased instrument longevity (Figure 2). Two panels—48 fixed LEDs each—provide fluorescent dye excitation over a broad spectrum. Each of the 48 wells is individually illuminated, optimizing the signal per well and minimizing cross-talk between wells.

The high-performance Eco optical system enables real-time detection of up to four targets in a single reaction. Four emission filters in a linear filter slide and a high-performance CCD camera detect the fluorescence from all wells, preventing any data loss and allowing changes to the plate setup and data analysis even after the run is completed. Standard melt curve and HRM analysis protocols support continuous data acquisition in a single dye channel during the melt for increased data collection and reduced run times.

Table 1: Reaction Mix for SYBR Assay and DNA Sample Specifications

Reagent	Amount (1 rxn)
2× SYBR Master Mix	5.0 µl
20× Human F5 Assay Mix	0.5 µl
Human Genomic DNA (2500 c/µl)	2.0 µl
Water	2.5 µl
Total Volume	10 µl

Table 2: Reaction Mix for FAM, HEX, and ROX Assays and DNA Sample Specifications

Reagent	Amount (1 rxn)
2× PCR Master Mix	5.0 µl
20× Human B2M Assay Mix (FAM, HEX or ROX labeled)	0.5 µl
Human Reference cDNA (5 ng/µl or 2.5 ng/ µl)	2.0 µl
Water	2.5 µl
Total Volume	10.0 µl

Table 3: PCR Thermal Protocol

Stage	Temperature (°C)	Time (min)	Acquisition
Activation	95	0:10:00	
PCR (×40 cycles)	95	0:00:15	
	60	0:01:00	×
Melt	95	0:00:15	
	55	0:00:15	
	95	0:00:15	on ramp

Gage R&R Study Parameters

Gage R&R studies are valuable Six Sigma tools for evaluating and identifitying variables in equipment and technician performance that could impact analyses. A Gage R&R study was performed to determine the uniformity of results generated by the Eco system. The study was conducted by two different operators using four randomly selected Eco systems, with the same experiment performed in triplicate (three plates) using four different real-time fluorescent chemistries (SYBR, FAM, HEX, and ROX).

The SYBR assay was performed with 5,000 copies of human genomic DNA template in a 10 µl reaction volume, while the FAM, HEX and ROX assays were performed with 5 ng and 10 ng of Human Reference cDNA templates in 10 µl reaction volumes (Tables 1 and 2). Each plate was run with the same PCR thermal profile as recommended by the master mix manufacturer (Table 3).

Results

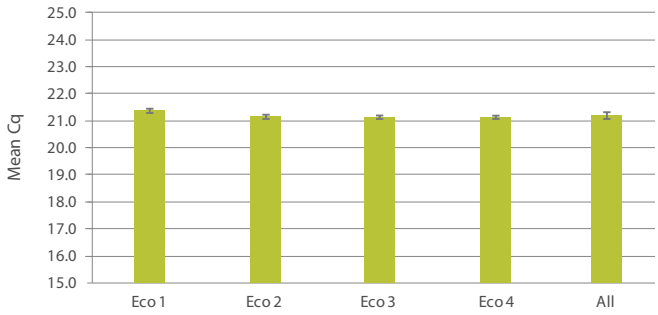
The Eco system demonstrated high precision within and between all four instruments (Figure 3). The mean Cq* (cycle of quantification) value for the SYBR assays was 21.17, with the standard deviation (SD) for each instrument ranging from 0.06 to 0.08. Such precision enables a statistically significant call to be made between samples with as little as 20% difference in target expression levels when run in duplicate in any position on the three plates. A Cq standard deviation value of 0.167 measured across all well positions indicates sufficient precision to discern a two-fold difference in starting target copy number with 99.6% confidence, and is a good measure of real-time performance. In our study, the precision between instruments is also high, with a 0.12 SD Cq for all four instruments. This value was also significantly below the 0.167 threshold and would enable detection of ~40% differences in starting target quantity among samples run in duplicate on any of the twelve plates run on any of the four instruments tested.

Similar precision can be seen in both the 5 ng and 10 ng samples using FAM-, HEX-, or ROX-based reporters. SD Cq values per plate for each of these fluores ranged from 0.05 to 0.11, while the SD Cq across all four plates for a given reporter was at or below 0.167. This data quality enables discrimination of two-fold changes in target expression level on any of the 12 plates run on any of the four instruments.

*Cq (also known as C_t) value is the cycle number when the amplification plot of the fluorescent signal crosses the threshold fluorescence value. Cq is the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) recommended unit for this point.

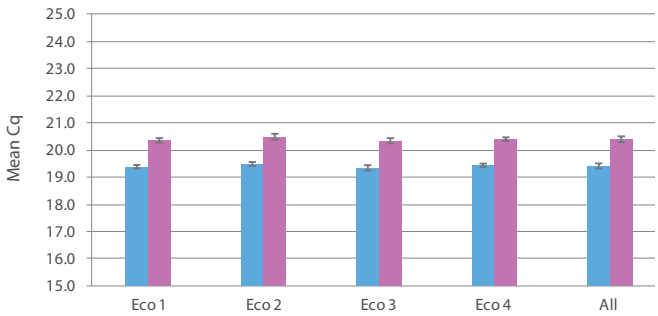
Figure 3: Gage R&R Results Demonstrating Eco System Precision

SYBR



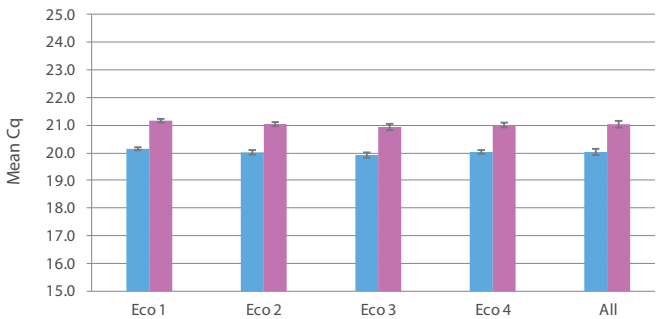
Cq Values	Eco 1	Eco 2	Eco 3	Eco 4	All
Mean Cq	21.346995	21.125142	21.110037	21.107388	21.172379
SD Cq	0.080465	0.080787	0.061504	0.060140	0.123704

FAM



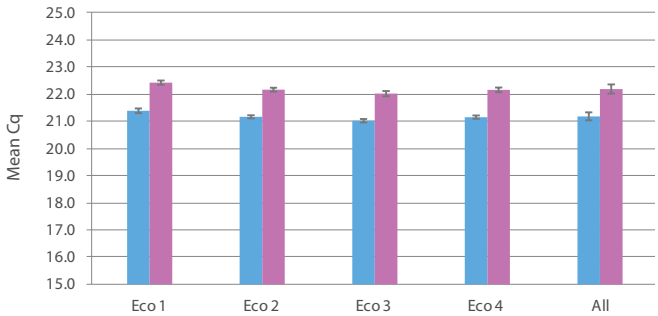
Cq Values	Eco 1	Eco 2	Eco 3	Eco 4	All
Mean Cq	19.368564	19.467332	19.327325	19.425172	19.397001
	20.338133	20.472526	20.324494	20.383787	20.379634
SD Cq	0.063577	0.077565	0.096859	0.062839	0.093273
	0.080992	0.104997	0.098314	0.070060	0.106312

HEX



Cq Values	Eco 1	Eco 2	Eco 3	Eco 4	All
Mean Cq	20.131977	20.003235	19.904437	20.017010	20.015409
	21.143987	21.020467	20.917064	20.989216	21.018062
SD Cq	0.050780	0.084583	0.095703	0.069449	0.111306
	0.065043	0.076403	0.114392	0.085479	0.119917

ROX



Cq Values	Eco 1	Eco 2	Eco 3	Eco 4	All
Mean Cq	21.362594	21.137223	20.995440	21.127214	21.155929
	22.393080	22.133101	21.987460	22.125101	22.157744
SD Cq	0.086624	0.057525	0.064525	0.063157	0.148916
	0.079171	0.069727	0.094665	0.083758	0.167696

The Gage R&R study was conducted by two different operators using four randomly selected Eco systems, with the same experiment performed in triplicate (three plates) using four different real-time fluorescent chemistries (SYBR, FAM, HEX, and ROX). SD Cq values ranged from 0.04 to 0.11, demonstrating high precision, and were substantially below the 0.167 threshold necessary to detect a two-fold difference in starting target copy number.

Implications for HRM Analysis

HRM analysis is a high-performance real-time PCR application that requires uniform temperature control and the collection of significantly more data points than usually provided for standard melt curve in order to detect subtle melting temperature (T_m) differences indicative of sequence variations within PCR amplicons. Mutations present in samples can be detected as either a shift in the T_m of the melt curve or as a change in the shape of the melt curve.

First designed for genotyping, HRM can be used to detect a small proportion of variant DNA in a background of wild-type sequence at sensitivities approaching 5%. Thermal precision is particularly important for the accurate genotyping of Class IV SNPs (A/T mutations), which occur at a frequency of ~7% within the human genome and are difficult to identify. The results of the Gage R&R demonstrate that the Eco system has the resolution necessary to measure the ~0.2°C T_m differences found between homozygous genotypes, with its uniform temperature control and precise fluorescence detection capable of delivering SD Cq values as low as 0.08.

In addition to genotyping, HRM analysis is now increasingly used for mutation screening and methylation analysis, where its resolution enables rapid, upfront screening prior to more in-depth analyses.

Conclusions

The Eco system delivers high well-to-well, plate-to-plate, and instrument-to-instrument uniformity, substantially reducing the need to run excessive technical replicates to generate statistical significance. These results support the use of the Eco system for high-performance applications such as HRM, where thermal precision is required to identify the subtle differences between genotypes.

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