Technical Overview

Genomics

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The Novel Optical Design of the Agilent Aria Real-Time PCR System Eliminates the Need for a Passive Reference Dye

Introduction

Real-time quantitative polymerase chain reaction (qPCR) detects and quantifies specific nucleic acid sequences by monitoring the increase of a fluorescent reporter after each amplification cycle, in real-time. The applications of qPCR are widespread, including gene expression analysis, detection of disease genes of interest, and more. The ability to rapidly and accurately detect minor changes among individual or multiplexed samples makes qPCR a powerful tool for molecular biology labs.

Over 90% of variability observed in qPCR data is a consequence of true, reproducible differences in the samples[1]. There can be several other sources of variability, which do not reflect a true, reproducible difference in the sample. Some nonreproducible variation in qPCR depends on the amplicon being measured, primers used, operator error, and error inherent to the system[2]. A significant amount of nonreproducible variability is caused by inherent errors, such as manufacturing differences in pipette tips, or the design of the qPCR instrument. Conventional qPCR instruments have inherent error arising from a stationary light source and fluorescent detector. Consequently, there is variation in raw fluorescent measurements for samples, depending on the location of the sample in the plate. Wells with longer, misaligned light paths, located on the perimeter of the plate, have a lower fluorescence reading than wells located in the middle of the plate, which have a shorter, direct light path (Figure 1).

Nonreproducible errors, such as those caused by a stationary optical system, can be minimized using a passive reference dye, such as carboxyrhodamine (ROX). The fluorescence from the ROX reference dye is not involved in the PCR reaction, nor affected by the target template; therefore, its emission intensity remains unchanged during the qPCR run. When the fluorescence emission intensity of the reporter dye is normalized to the fluorescence emission intensity of the ROX passive reference dye, nonreproducible variation is minimized.

In contrast to traditional qPCR systems, the Agilent Aria Real-Time PCR systems are designed to limit nonreproducible error with a unique optical system. The Aria uses solid-state modular optical filters, each with eight LEDs and photodiodes (Figure 2). The optical modules are loaded into the optical scanning module, which scans across the plate. Each LED and detector is centered above the well so the light path is identical for all wells across the plate. The consistent and direct light path for each sample limits significant sources of nonreproducible error.

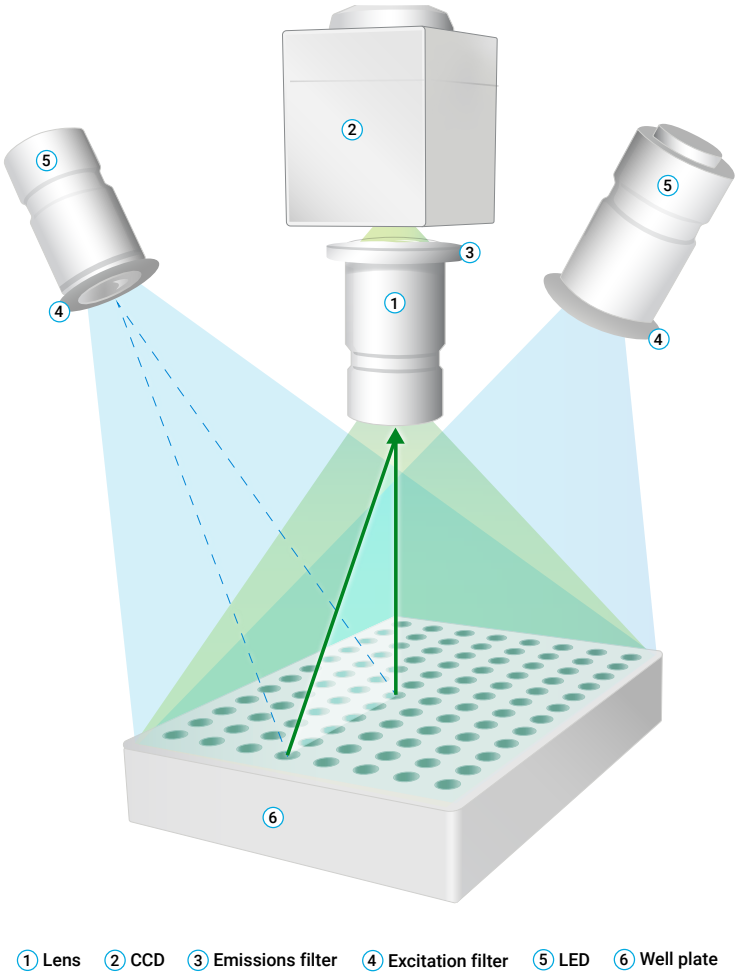


Figure 1. Schematic of the optical system configuration for conventional qPCR instruments. Wells in the center of the plate have a shorter, aligned light path, emitting a higher fluorescence signal, than those in the perimeter, which have a longer, misaligned light path. This results in the need for a passive reference dye in these optical systems to mitigate differences in raw fluorescence signals from nonreproducible error.

Another way the Aria minimizes nonreproducible error is with multiple optical filters which allow for multiplexing qPCR. Multiplexing qPCR analyzes multiple targets within a single reaction, providing internal controls, reducing costs, and preserving precious samples. The number of targets which can be analyzed in a sample is determined by the number of channels available for fluorescent detection. Using a passive reference dye limits the capacity of a qPCR instrument, as it occupies an optical channel that could be used for detecting a target of interest.

The Aria allows users to select from a variety of optical modules for custom detection of up to five different targets in a single sample. The optical modules can be switched between experiments and instruments, and additional optical

Figure 2. The Agilent Aria Real-Time PCR systems use optical modules with eight LEDs and detectors, which are centered above each well in a plate for identical light paths across the plate. This unique design eliminates the need for a passive reference dye.

module types can be purchased at any time to accommodate diverse qPCR applications. In this technical note, we assess the internal error of the Aria system by measuring the percent coefficient of variation (CV) across quantitative cycles (Cq) for samples with and without a passive reference dye.

Methods

The experiments in this study were performed with the Agilent AriaMx Real-Time PCR instrument (p/n G8830A) using the SYBR/FAM (p/n G8830-67001) and ROX (p/n G8830-67002) optical modules. Data was collected and analyzed with the AriaMx software version 1.8. The DNA template was obtained by diluting human genomic DNA (Promega p/n G3041) in nuclease-free water to achieve final concentrations of 100 ng/µL, 50 ng/µL, 25 ng/µL, and 12.5 ng/µL. Primers for both TATA-binding protein (TBP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicons were obtained from IDT. DNA amplification was achieved using the Agilent qPCR Brilliant III SYBR master mix (p/n 600883), Agilent AriaMx semi-skirted 96-well plates (p/n 401333) and Agilent AriaMx adhesive plate seals (p/n 401492) following standard protocols. Each amplicon (TBP and GAPDH) was analyzed both with and without ROX reference dye, included in the Brilliant III SYBR master mix kit. Two different plate layouts were used in this technical note. In one layout, each gDNA concentration was distributed across three columns. Each row was identical and contained triplicates of the DNA template dilutions, for a total of 24 replicates per concentration (Figure 3A). The other plate layout consisted of a single dilution of 50 ng/µL in all 96 wells (Figure 3B).

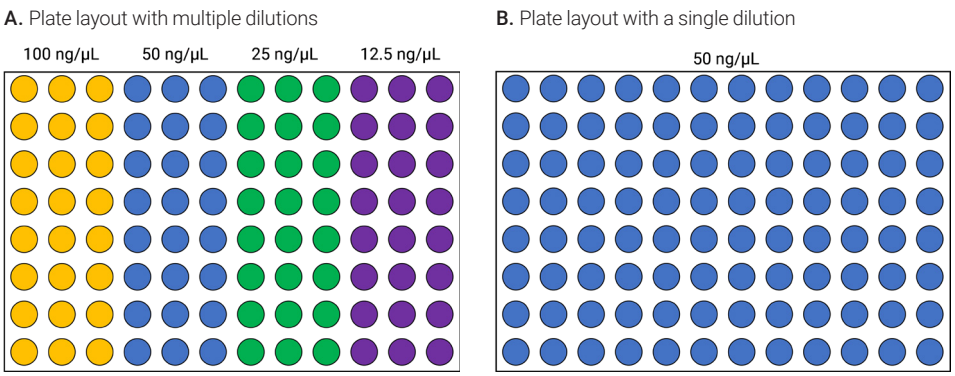


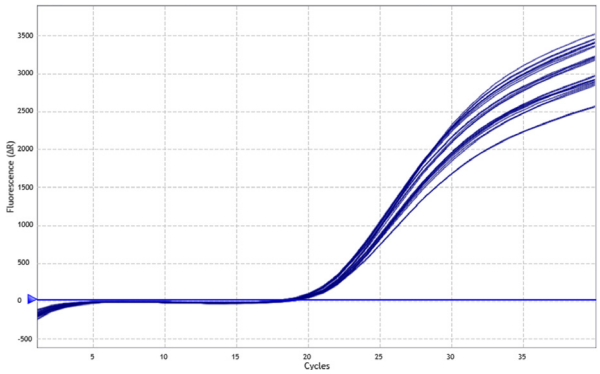
Figure 3. (A) Plate layout with triplicates of each human genomic DNA dilution. Dilutions of 100 ng/µL (yellow), 50 ng/µL (blue), 25 ng/µL (green) and 12.5 ng/µL (purple) of human gDNA are spread across three columns. Each row is identical, with each dilution of the DNA template distributed in triplicate. (B) Plate layout with 50 ng/µL dilution of human genomic DNA in every well.

Results and discussion

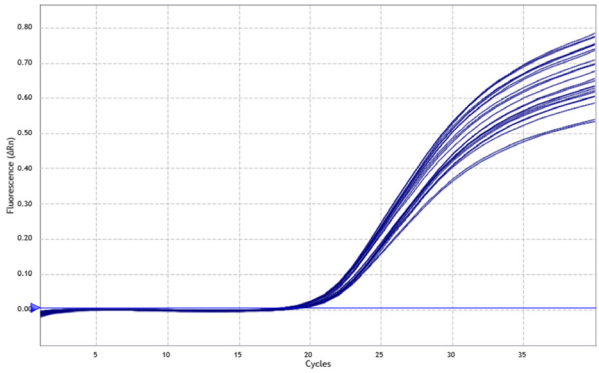
Comparison of quantitation cycle (Cq) with or without reference dye

To assess the reproducibility of the Aria instrument, the average, standard deviation (SD), and percent coefficient of variation (CV) of the Cq was examined. Since the Cq is directly related to the initial concentration of the DNA template, a dilution series (Figure 3A) was analyzed for both amplicons with and without ROX. Additionally, the size of the amplicon can affect the reproducibility of the Cq. The GAPDH amplicon is 92 bp longer than the TBP amplicon, and is expected to have a larger percent CV. In all conditions, the amplification plots from replicates of each dilution cross the threshold at nearly the same Cq, indicating low variation between the replicates. Figure 4 shows representative amplification plots from 50 ng/µL for each condition. The remaining three dilutions (100 ng/µL, 25 ng/µL, and 12.5 ng/µL) had similar results (data not shown).

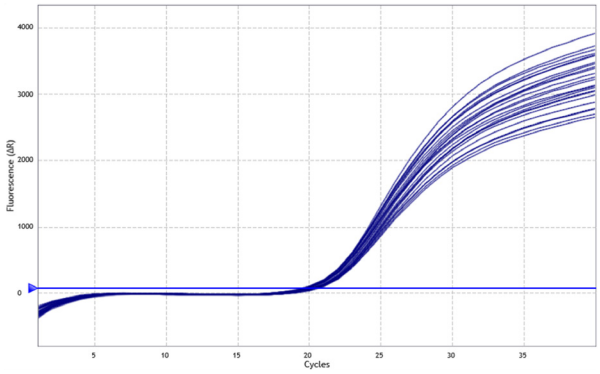
A. GAPDH 50 ng/µl



B. GAPDH 50 ng/µl including ROX



C. TBP 50 ng/µl



D. TBP 50 ng/µl including ROX

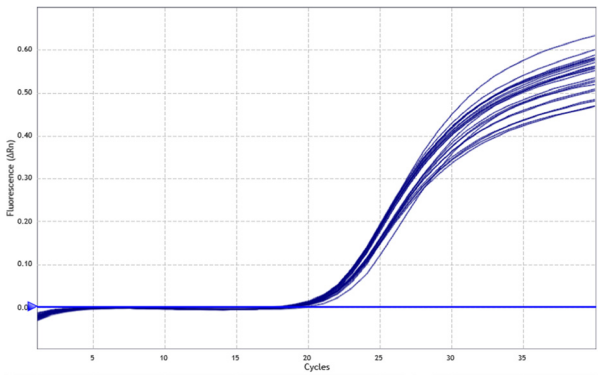


Figure 4. Representative amplification plots. Amplification plots from all replicates of 50 ng/µl human genomic DNA (A) GAPDH amplicon, (B) GAPDH amplicon with ROX, (C) TBP amplicon, and (D) TBP amplicon with ROX. The replicates for each condition cross the threshold (blue horizontal line) at a similar Cq: 20.34, 19.57, 21.23, and 20.16, respectively.

As expected, each amplicon had different variability based on the gene being amplified, the primers being used, and the length of the amplicon. The GAPDH amplicon (Table 1) resulted in a larger percent CV than the TBP amplicon (Table 2) with and without ROX. Additionally, the samples analyzed with the reference dye did not consistently have the lowest percent CV. The two GAPDH amplicon dilutions, 25 ng/µl and 12.5 ng/µl, had lower percent CV with the reference dye than without (Table 1). The TBP amplicon had only one dilution, 100 ng/µl, with a lower percent CV with the reference dye than without (Table 2). This demonstrates that the Aria delivers reproducible results, with or without a reference dye. The low percent CV without the use of the ROX passive reference dye indicates that the design of the Aria Real-Time qPCR system minimizes nonreproducible error.

To assess the precision of the Aria system, the average, standard deviation and percent CV was analyzed from a plate with 50 ng/µl starting DNA template in every well (Figure 3B). A single plate containing ROX was prepared and the fluorescence from both the target fluorophore (SYBR) and the passive reference dye (ROX) was measured. The Cq of the SYBR fluorophore was analyzed with the AriaMx software, both with and without normalizing to ROX. The consistency of the Cq values is demonstrated in a three-dimensional surface plot (Figure 5). The plate layout is plotted on the X (column) and Z (row) axes, while the Cq value for each well is plotted on the Y-axis. Similar Cq values result in a flat surface plot. The percent CV from both plates was below 2% (Table 3). The Cq, standard deviation, and percent CV, both with and without ROX is nearly identical, demonstrating that the Aria system does not require the use of the ROX passive reference dye for reproducible results.

Table 1. Comparison of the average Cq, standard deviation, and percent CV for all replicates of each dilution for the GAPDH amplicon with and without the reference dye.

Starting GAPDH DNA (ng/µl)	Reference Dye	Average Cq	Standard Deviation	%CV
100	None	19.78	0.39	1.97%
50	None	20.34	0.29	1.43%
25	None	21.15	0.53	2.51%
12.5	None	21.75	0.64	2.94%
100	ROX	18.86	0.51	2.70%
50	ROX	19.57	0.46	2.35%
25	ROX	20.43	0.36	1.76%
12.5	ROX	21.41	0.24	1.12%

Table 2. Comparison of the average Cq, standard deviation, and percent CV for all replicates of each dilution for the TBP amplicon with and without the reference dye.

Starting TBP DNA (ng/µl)	Reference Dye	Average Cq	Standard Deviation	%CV
100	None	20.87	0.35	1.68%
50	None	21.23	0.31	1.46%
25	None	22.58	0.20	0.89%
12.5	None	23.40	0.20	0.85%
100	ROX	19.58	0.29	1.48%
50	ROX	20.16	0.35	1.74%
25	ROX	20.44	0.21	1.03%
12.5	ROX	21.61	0.22	1.02%

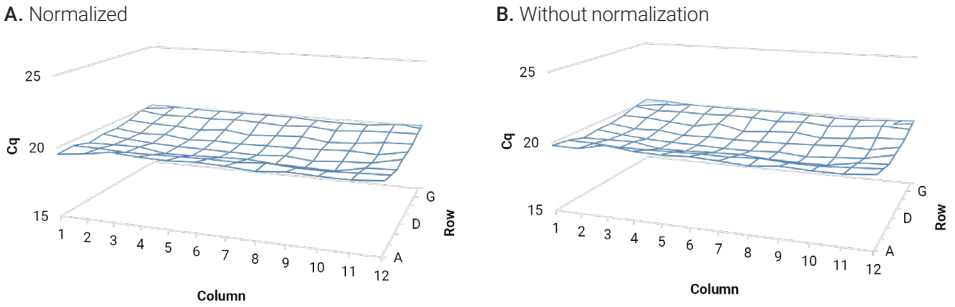


Figure 5. Three-dimensional surface plots of a single plate where each well contained 50 ng/µL and ROX. The analysis of the Cq was completed with A) normalization to ROX and B) without normalization to ROX. Both plots have similarly flat surfaces, which indicate similar Cq values in every well, with or without ROX.

Table 3. Comparison of the Average Cq, standard deviation, and percent CV of a single 50 ng/µL dilution for every well in a plate. Each reaction contained ROX, and the analysis was performed both with and without normalization.

	Average Cq	Standard Deviation	%CV
Normalized	19.51	0.32	1.65%
Without Normalization	19.53	0.35	1.79%

Conclusion

The need to quickly discriminate between several diseases such as SARS-CoV-2, influenza A and B, and other respiratory illnesses, has increased the demand for qPCR analysis of multiple targets in a single sample. However, the number of targets which can be analyzed in a sample is limited by the available channels for fluorescent detection. When the need for a reference dye is eliminated, an additional channel becomes available. For traditional qPCR instruments, normalization to a reference dye is required to reduce nonreproducible error caused by lengthy indirect light paths within the optical system.

The Agilent Aria Real-Time PCR systems use a moving optical scanning module which eliminates signal variability from well-to-well differences in the light path of each sample to the detector. This unique optical design ensures that every light path to the detector is identical, minimizing nonreproducible error, eliminating the need for a passive reference dye (ROX), and providing flexibility in the simultaneous detection of multiple targets in a single reaction. In this technical overview, we demonstrate that the Aria systems can provide precise analysis with or without the use of ROX as a passive reference dye. Users can accurately analyze up to five different targets in a single sample with the Aria system for increased productivity, and reduced costs.

References

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