Azure Cielo™ Real-Time PCR Optical Design Eliminates the Need for Passive Reference Dye

Introduction

ROX, also known as carboxyrhodamine, is a widely used inert fluorescent dye that is commonly added to a qPCR reaction mixture to normalize the fluorescent signal.[1] However, with the advancement of fluorescent technology in instruments such as the Azure Cielo Real-Time PCR systems, the extra step of using a separate dye solely for the purpose of normalization is no longer necessary.

When real-time PCR systems were first introduced to the market, many used a stationary light source and detection system to image all 96 wells simultaneously, resulting in varying optical paths for each well in the thermal block (Figure 1). These optical path differences would cause variations in the absolute fluorescence measurements of each well depending on where they were located on the plate. For example, a well with a shorter optical path could appear to have a higher fluorescence reading than a well with a longer optical path, regardless of the actual fluorescence of that well at that point in the cycle. Because of this, many researchers began normalizing for this signal variation using a reference dye – such as ROX.

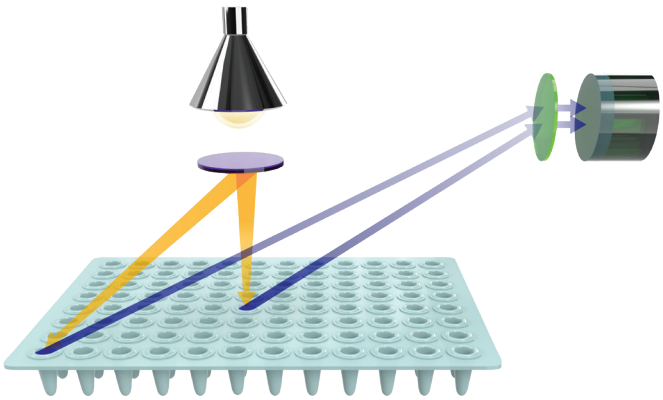

Figure 1. Schematic representation of whole plate imaging using a conventional Real-Time PCR system. The well in the center of the block has a shorter light path (yellow) and therefore emits a higher fluorescence signal compared to the well at the corner (blue), which has a longer light path.

Because the ROX signal is not affected by the qPCR reactions, it remains stable throughout the entire qPCR process, provided the attributes of the qPCR mixture are kept constant. It was for this reason – and because ROX has superior spectral properties compared to other dyes – that ROX was seen as a good candidate to normalize the fluorescent signal and account for the varying optical paths in real-time PCRs utilizing single-lamp technology.

The challenge with using ROX as a reference dye is that it eliminates a channel that could potentially be used for detecting a target of interest. With applications such as detection of Influenza and COVID-19 gene targets (see ANDiS SARS-CoV-2 and Influenza A/B RT-qPCR Detection Kit) becoming more common, 5 plex dye reactions are increasingly routine; this highlights the need for an alternative solution.

Azure Cielo Real-time PCR systems use a fiber optic detection system that negates the requirement to include ROX as a passive dye in a qPCR experiment. Rather than a single, stationery light source and detector system, Cielo Real-Time PCR systems utilize a 16-well simultaneous excitation and signal detection that ensure uniform data acquisition (Figure 2). Additionally, within each well, the system scans approximately 100,000 pixels worth of data (Figure 3); this “total well detection” produces data that is more reproducible and reliable than a system that scans only a few pixels per well.

In this study, we confirm the uniform detection of the Cielo Real-Time PCR systems. Our results demonstrate that, without ROX, Cielo Real-Time PCR systems generate C_q values in ten technical replicates with a standard deviation as low as 0.02, rendering the use of ROX for normalization unnecessary.

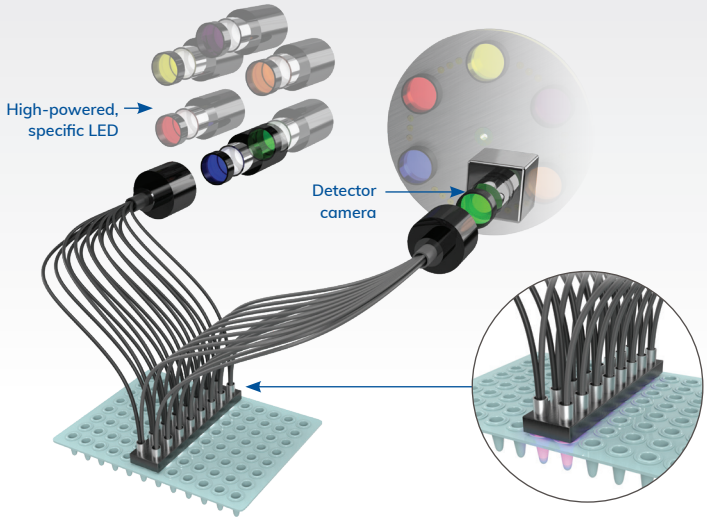

Figure 2. Optics of the Cielo 6 Real-time PCR system. The optics system comprises two sets of 16 fiber optics, one providing the excitation light (dark grey) and one to collect the emitted light (light grey). 16 wells are detected simultaneously, with each well individually excited by a high-powered Channel Specific LED and detected on a per well basis by the camera. The Cielo 3 Real-Time PCR system has 3 LED light sources and 3 detection filters.

Methods

The real-time PCR quantification was performed in ten replicates, at a 20 µL final reaction volume per well. It comprised 5 µL human 2X genomic DNA (10 ng/µL stock concentration), 1 µL primer pairs, 10 µL no ROX qPCR mix and 4 µL qPCR grade water. A concentration of 0.2 mg/mL human genomic DNA (#11691112001, MilliporeSigma) was diluted to 10 ng/µL to serve as the nucleic acid template. Primer pairs (PrimerPCR™ SYBR® Green Assay, Bio-Rad Laboratories Inc) designed for human GAPDH gene detection (#qHsaCED0038674) and qMAX Gold No ROX qPCR mix (PR2010-N-500, Accuris Instruments) were used to prepare the qPCR reaction mixture. A master mix was prepared such that an identical amount of mix was pipetted into semi-skirted low-profile qPCR tubes (0.1 mL) and run on a Cielo 3 Real-Time PCR system. Thermal cycling was set to one cycle for 3 min at 95°C, then 40 cycles for 10 sec at 95°C and 30 sec at 60°C.

Results and Discussion

Results show that the Cielo Real-Time PCR system can uniformly detect amplifications in every qPCR well. The real-time amplification curves from all ten replicate wells (Figure 3B) show a uniform increase of signal detected, giving a C_q mean value of 17.54 with standard deviation as low as 0.02 from the statistical analysis (Table 1). The coefficient of variation (CV) of C_q values is 0.13%, reflecting the excellent precision of the data. In combination, the fiber optic detection system and the Marlow Peltier heating block achieve inter-well signal detection uniformity and heating temperature uniformity in the qPCR experiment and ensure low background signal since each sample is excited and scanned individually. The benefits of these features are demonstrated by the incredibly low standard deviation and CV values for the ten replicate reactions, denoting sensitive and reproducible qPCR results across all wells without the need for ROX as a passive dye reference.

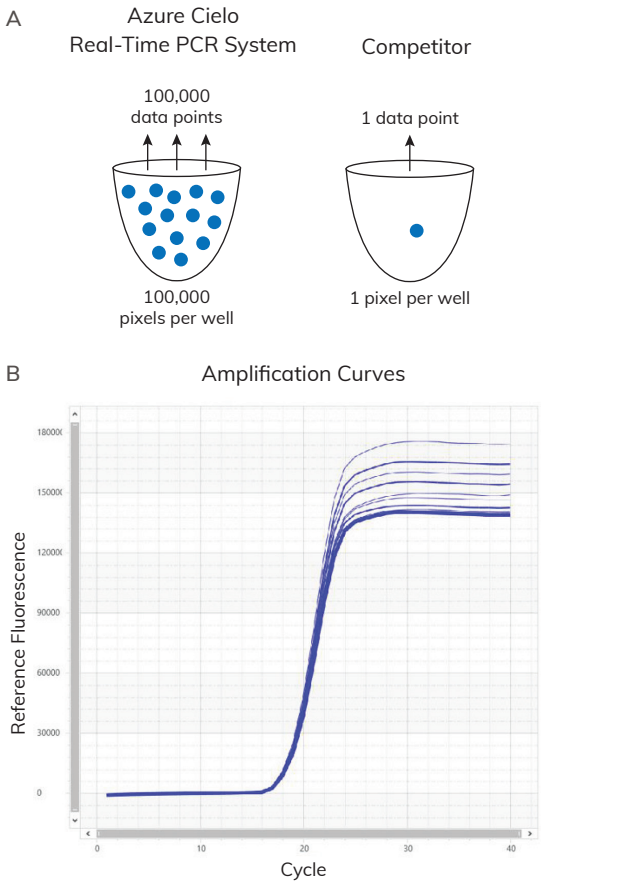

Figure 3. Optical design eliminates the need for ROX. A) Cielo Real-Time PCR system total well detection acquires approximately 100,000 data points per well, with readings then averaged to precisely determine the fluorescent intensity in each well. In contrast, competitor PCR systems capture only a handful of data readings per well, providing a less accurate picture of total well values. B) Amplification curves generated using a human genomic DNA template with human GAPDH primers and a no ROX SYBR Green qPCR mix.

Replicate	Fluorescence	C_q	C_q Mean	C_q Standard Deviation	C_q CV
1	SYBR Green (NO ROX)	17.54	17.54	0.02	0.13%
2		17.57			
3		17.55			
4		17.57			
5		17.56			
6		17.54			
7		17.52			
8		17.52			
9		17.51			
10		17.51			

Table 1. Results generated using the Cielo Real-Time PCR system for reactions with no passive reference dye added. The C_q values were obtained from ten replicate wells and the respective statistical data is shown.

Conclusion

Using ROX in a qPCR reaction is helpful in correcting signal variations in instruments that utilize a stationary light source and detection system with whole plate imaging technology. In this experiment, we confirm that Cielo Real-Time PCR systems provide uniform detection and superior gene quantification without the use of ROX as a passive reference dye. The 16-well fiber optic system shows minimal variation within the ten replicate wells, eliminating the previously commonplace signal variability caused by optical path variation to provide precise and reproducible qPCR data every time, without the use of extra dyes.

Reference

1. Slateva, K., Elsner, H.-A., Albis-Camps, M., & Blasczyk, R. (2001). HLA-DRB fluorotyping by dark quenching and automated analysis. *Tissue Antigens*, 58(4), 250-254. https://doi.org/10.1034/j.1399-0039.2001.580405.x

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