Sensitive and Accurate Single–Copy Amplicon Detection in Azure Cielo Real–Time PCR System

Introduction

The Azure Cielo 3 and Cielo 6 Real-Time PCR systems are designed for optimal sensitivity. The innovative optics in these instruments comprise two sets of 16 optical fibers, one set for high-powered LED excitation and one for detecting emission for each of 16 wells (Figure 1). This allows individual-well illumination, with uniform illumination of the entire well and elimination of light scatter as a source of background noise for more sensitive detection.

Detection of emission by the Cielo Real-Time PCR systems' CMOS camera involves capturing approximately 100,000 pixels or data points per well. This "total well detection" provides more accurate data for each collection time point than single photo-detector systems which essentially record one measurement or data point per well. In combination with the lower noise provided by the single-well excitation and detection optics, the Cielo Real-Time PCR systems provide high intra-well reproducibility for the most reliable data.

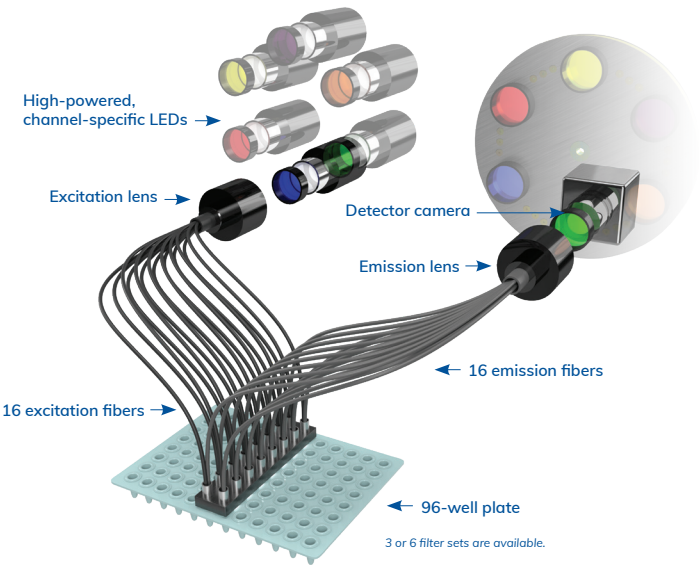

Figure 1. Innovative Cielo Real-Time PCR system optics.

The ability to reliably detect very low target copy numbers depends on several factors. These include the assay efficiency, which hinges on the specificity of the primers and probes, and the sensitivity of the instrument to detect when the probe signal increases above background noise. With all other reaction components being equal, higher-sensitivity equipment will enable earlier detection or earlier C_q values, translating to fewer PCR cycles needed and faster experiments.

To demonstrate sensitivity and reproducibility at low target amounts, the Cielo Real-Time PCR system was used to detect amplification of a single copy of target DNA.

Method

A master mix containing PCR components and one copy of target DNA sequence per 20 µl reaction was prepared and identical 20 µl reactions pipetted into a 96-well plate. The final composition of each reaction was:

- 1x iQ™ Multiplex Powermix (Bio-Rad 1725848)
- 1x PrimePCR™ GAPDH Probe Assay FAM (Bio-Rad)
- 1 copy PrimePCR™ Template for Probe Assay, GAPDH Human (Bio-Rad)

Plates were sealed and PCR carried out according to the following protocol:

1. Initialization at 95°C for 1 min 30 sec
2. Denaturation at 95°C for 15 sec
3. Annealing and extension at 60°C for 20 sec followed by a plate read 50 cycles of Steps 2 and 3.

Duplicate plates were run, one on the Cielo Real-Time PCR system and the other on a competitor system.

Data were collected and analyzed using the software provided by each instrument to determine the C_q for each well.

Results and Discussion

Single copy amplification was detected in 88 wells (92%) of the plate run on the Cielo Real-Time PCR system, and in 77 wells (80%) on the plate run on the competitor system (Figure 3). With single copy reactions, there is a probability that no target will make it into some wells.[1] In addition, very late increases in fluorescence may be seen in some reactions, as observed during testing carried out on the competitor machine (Figure 2B). The high sensitivity "total well detection" of the Cielo Real-Time PCR system with multiple data point capture increases the probability of detecting single copy amplification.

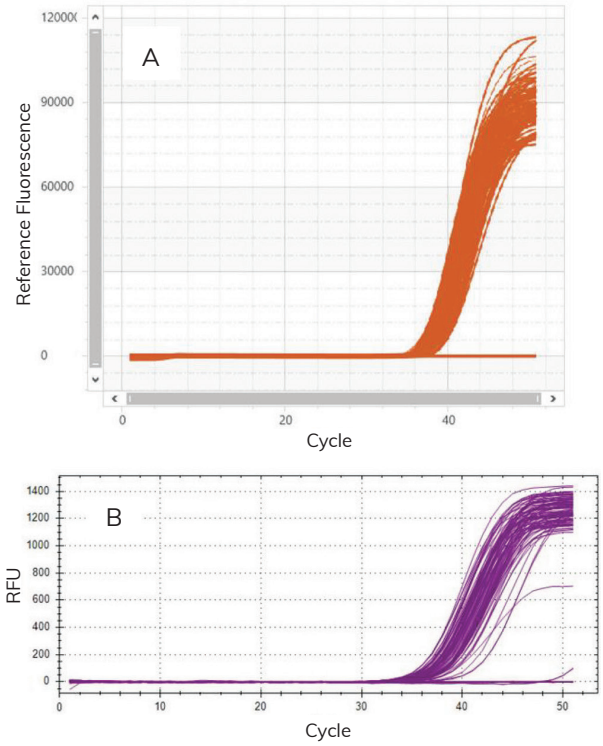

Figure 2. Amplification curves for 96 replicates of single-copy qPCR reactions carried out on (A) the Cielo Real-Time PCR system or (B) a competitor PCR system.

Single copy amplification was detected an average of two cycles earlier with the Cielo Real-Time PCR system compared to the competitor (C_q 36.39 vs 38.53) (Table 1, Figure 2). Additionally, the standard deviation for C_q measured with the Cielo Real-Time PCR system was smaller than that observed with the competitor (0.91 vs 1.16), representing excellent reproducibility among replicates. qPCR is known to become less reliable with decreasing target amount,[2] yet modern applications continue to push the limit of the technology, demanding ever higher performance.[3,4]

These results demonstrate the superior sensitivity and reliability of the Cielo Real-Time PCR system, which provides reproducible quantitation down to a single copy of the target sequence.

	Positive wells, n (%)	C_q (mean)	C_q (StDev)	C_q competitor–C_q Cielo
Cielo	88 (92)	36.39	0.91	2.14
Competitor	77 (80)	38.53	1.16	

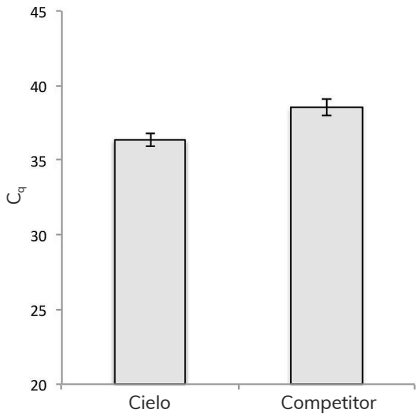

Figure 3. Superior Single Copy Detection. Average C_q obtained from Azure Cielo System is 2 cycles earlier than Competitor. Error is standard deviation.

References

1. Lockey C, Otto E, Long Z. Real-Time Fluorescence Detection of a Single DNA Molecule. *BioTechniques.* 1998; 24:744–746.
2. Stowers CC, Haselton FR, Boczko EM. An Analysis of Quantitative PCR Reliability Through Replicates Using the Ct Method. *J Biomed Sci Eng.* 2010;3(5):459-469.
3. Stadler J, Eder J, Pratscher B, et al. SNPase-ARMS qPCR: Ultrasensitive Mutation-Based Detection of Cell-Free Tumor DNA in Melanoma Patients. *PLoS One.* 2015;10(11):e0142273.
4. Tosiano MA, Jacobs JL, Shutt KA, et al. A Simpler and More Sensitive Single-Copy HIV-1 RNA Assay for Quantification of Persistent HIV-1 Viremia in Individuals on Suppressive Antiretroviral Therapy. *J Clin Microbiol.* 2019;57(3):e01714-e01718.