6-Channel Multiplex qPCR Using Azure Cielo Real-Time PCR System

Introduction

Multiplex real-time PCR offers several advantages for investigators studying more than one target of interest. First, assaying multiple genes per PCR run saves both time and money, providing results more quickly while minimizing consumption of consumables such as PCR tubes or plates. Additionally, more information is obtained per sample, which can especially be important when sample amounts are limited. Lastly, because RNA levels or gene copy numbers are compared directly within the same reaction, controls to account for pipetting differences between wells or plates are not required.

The use of target-specific probes with different fluorophore labels in multiplex real-time PCR allows detection of each product in a different fluorescent channel. This eliminates the need for PCR products to be differently sized, in contrast to end-point multiplex PCR where products are analyzed by gel electrophoresis. Moreover, reactions using target-specific fluorescent probes are not subject to interference from nonspecific signal that can arise from secondary PCR products binding to intercalating dyes such as SYBR Green.

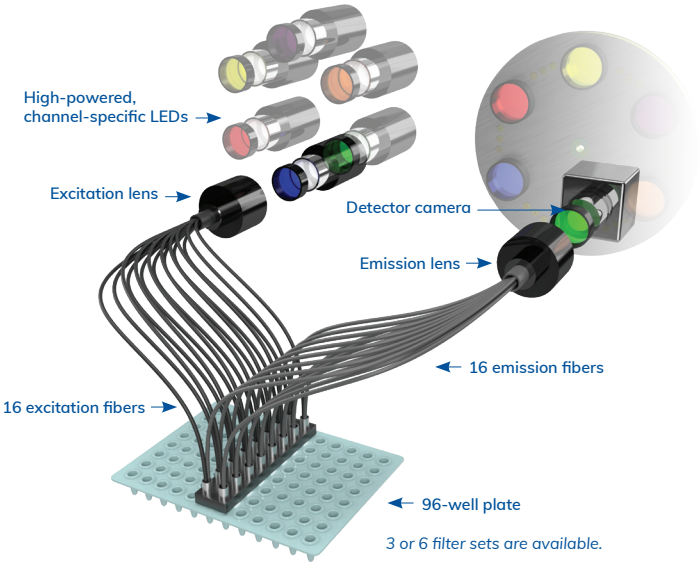


Figure 1. Azure Cielo innovative and high-performance optical technology. Two sets of 16 optical fibers allows 16 individual wells to be imaged simultaneously for faster and efficient scan times.

Multiplex real-time PCR has many applications, from the study of gene expression to the rapid detection and differentiation of potential pathogens in a biological sample.[1–3] The technique is so powerful, it has even been optimized for use in microgravity on the International Space Station.[4] Much recently, multiplex qPCR has been widely used in SARS-CoV-2 and Influenza A and B differentiation[5].

The Azure Cielo Real-Time PCR systems are designed for multiplex experiments involving up to six different targets. Innovative optical technology with two sets of 16 optical fibers allows 16 individual wells to be imaged simultaneously, meaning an entire 96-well plate can be imaged in all six fluorescent channels in just nine seconds (Figure 1). Additionally, the Azure Cielo Real-Time PCR system is designed to scan up to 6 individual fluorescent channels that covers most of the qPCR chemistry spectrum (Table 1). This is complemented by an individual-well scanning system which reduces noise by eliminating the light scatter that can occur when a single light source is used to illuminate a full plate or multiple wells (Figure 2). The single-well scanning system also reduces the opportunity for inter-well crosstalk.

To demonstrate the multiplex performance of the Cielo Real-Time PCR systems, we conducted a six-plex one-step reverse transcription quantitative PCR (RT-qPCR) assay on a dilution series of human cDNA.

Dyes/Probes	Azure Cielo 3 Real Time PCR System	Azure Cielo 6 Real Time PCR System
SYBR® Green, EvaGreen™, FAM™	✓	✓
VIC®, HEX™, JOE™, CAL Fluor® 540, CAL Fluor® Orange 560	✓	✓
TAMRA™, Cy3		✓
ROX™, TEX®615, CAL Fluor® Red 610		✓
Cy®5, Quasar®670, Liz®, Mustang Purple®	✓	✓
Cy®5.5, Quasar 705		✓

Custom dyes and probes are compatible based on system configuration.

Table 1. Azure Cielo Real-Time PCR system is compatible with a wide variety of qPCR chemistries. This allows broad compatibility with a variety of qPCR assays.

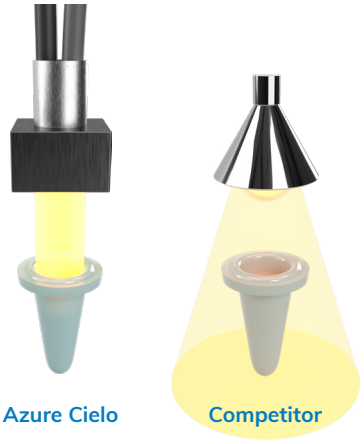


Figure 2. Individual well scanning eliminates the light scatter than can occur with whole-plate illumination or detection. Additionally, illumination and detection of individual wells improves data consistency across wells and experiments.

Method

A six-plex RT-qPCR reaction was designed to simultaneously detect six mRNAs in a sample of human cDNA. Standard curves were generated for each of the six genes by performing a cDNA dilution series. The genes assessed and primer/probe assays used are listed in Table 1.

The target for the six-plex reaction was a two-fold dilution series of Human Reference cDNA (Takara Bio) ranging from 250ng to 1.95ng per reaction. Each dilution was assayed in duplicate.

Target (all human)	Assay	Probe
Ribosomal RNA processing 36 homolog (RRP36)	Bio-Rad qHsaCIP0029701	FAM
Beta actin (ACTB)	Bio-Rad qHsaCEP0036280	HEX
RNase P	Bio-Rad #12004602	TAMRA
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Bio-Rad qHsaCEP0041396	TEX 615
TATA box binding protein (TBP)	Bio-Rad qHsaCIP0036255	Cy5
Eukaryotic elongation factor 1 alpha 1 (EEF1A1)	Bio-Rad qHsaCEP0052990	Cy5.5

Table 2. mRNA targets assessed.

A master mix solution was prepared such that each 20 µl reaction contained a final concentration of 1X Multiplex Supermix (Bio-Rad) and 0.5 µl of each of the six primer/probe assay mixes.

After sealing the PCR plate, PCR was conducted 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 45 cycles of steps 2 and 3.

Data were extracted and analyzed using the Azure Cielo Manager's Absolute Quantification Mode to identify the C_q and generate a standard curve for each of the six channels.

Results and Discussion

The amplification curves in each of the six channels are shown in Figure 2. No evidence of signal leakage or crosstalk were observed in the single-channel amplification curves.

The efficiencies and linear dynamic range for each of the reactions in the six-plex experiment are shown in Figure 3. Each reaction showed an excellent efficiency near 100% and R^2 values close to 1 across the range of target cDNA used.

These results demonstrate the exceptional performance of the Cielo Real-Time PCR system in carrying out six-plex real-time PCR reactions. With single-well scanning, no evidence of leakage or crosstalk is seen between any of the channels. The low background and consistent, reproducible illumination produce clean data with highly linear standard curves.

The six fluorescent channels available on the Cielo 6 provide the ultimate flexibility in experimental design, with the ability to assess six targets simultaneously and to use a wide variety of commercially available or custom probes. To learn more about how Cielo Real-Time PCR systems can enhance the performance of your assays, visit https://www.azurebiosystems.com/cielo-6/.

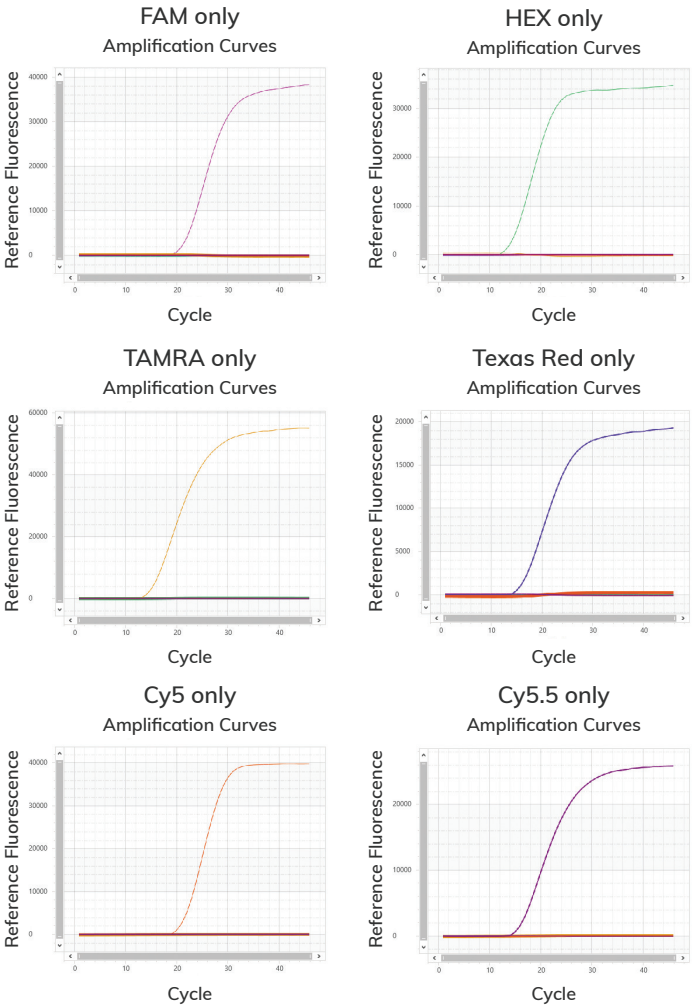
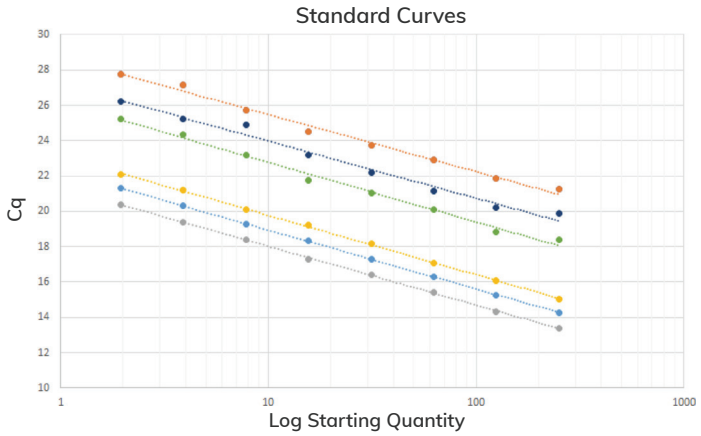


Figure 3. Multiplex RT-qPCR reactions were scanned for all 6 channels on a Cielo 6 Real-Time PCR system. No evidence of signal leakage or crosstalk is seen in the single-channel amplification curves.



Report Dye	Gene name	Efficiency	R^2	Slope	Y-intercept
FAM	RRP36	103.582	0.959	-3.239	28.684
HEX	ACTB	100.456	0.995	-3.311	21.281
TAMRA	RNASE-P	97.016	0.995	-3.396	22.157
Texas Red	GAPDH	100.219	1.000	-3.317	22.307
Cy5	TBP	96.811	0.993	-3.401	26.023
Cy5.5	EF1a	97.224	0.993	-3.390	23.173

Figure 4. The linear dynamic range for each reaction in the six-plex one-step reverse transcription qPCR experiment. Excellent efficiencies and linearity over a wide dynamic range were observed for each assay.

References

1. Valasek MA, Repa JJ. The power of real-time PCR. *Adv Physiol Educ.* 2005;29:151–159.
2. Shin JH, Lee SE, Kim TS, et al. Development of molecular diagnosis using multiplex real-time PCR and T4 phage internal control to simultaneously detect Cryptosporidium parvum, Giardia lamblia, and Cyclospora cayetanensis from human stool samples. *Korean J Parasitol.* 2018;56(5):419–427.
3. Irshad M, Gupta P, Mankotia DS, et al. Multiplex qPCR for serodetection and serotyping of hepatitis viruses: A brief review. *World J Gastroenterol.* 2016;22(20):4824–4834.
4. Parra M, Jung J, Boone TD, et al. Microgravity validation of a novel system for RNA isolation and multiplex quantitative real time PCR analysis of gene expression on the International Space Station. *PLoS ONE.* 2017;12(9):e0183480.
5. FDA. (2020). CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay. FDA.Gov. https://www.fda.gov/media/139743.

MM-0106 R1