Detection of low copy telomeric genes with the Azure Cielo™

Introduction

Quantitative real-time PCR (qPCR) is a powerful technology that has proved invaluable to basic research across a broad range of fields as well as to diagnostics. The technique involves measuring the fluorescence signal of a probe that fluoresces when it binds to the PCR product, allowing product accumulation to be followed in real-time. The point at which the fluorescence signal crosses above a predetermined background threshold level is called the quantification cycle, or Cq, and is proportional to the amount of target in the starting reaction. Therefore, the relative number of copies of target sequence between samples can be determined in a semi-quantitative way by comparing Cq values. The method is fully quantitative and the exact number of initial copies in a sample can be determined by using standard curves.[1]

The Cq value is related to the amount of target sequence in the sample but will be influenced by the efficiency of the PCR reaction and also by the sensitivity of the instrument to detect fluorescence. The sensitivity of the instrument can reduce the number of cycles needed to detect product, saving time and increasing efficiency. The Azure Cielo is designed for high performance with individual excitation and emission fibers for each well, increasing sensitivity and reducing background noise (Figure 1). In addition, the Cielo captures approximately 100,000 pixels per well instead of a single pixel per well as some competitors (Figure 2).

In this technical note, the sensitivity of the Cielo was compared to that of a competitor instrument by comparing the Cq values observed with duplicate reactions run on the two instruments. Three yeast RNA targets were studied, representing a range of expression levels including very high (actin mRNA), low (telomeric non-coding RNA (TERRA) originating at 6 Y' elements in the subtelomeric region), and very low (a single-copy TERRA target from TEL06R, the telomeric region of the right arm of chromosome 6).

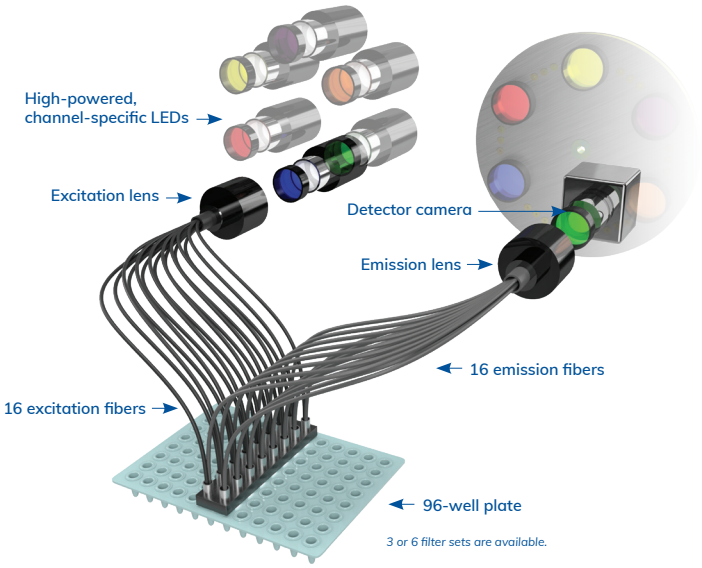

Figure 1.

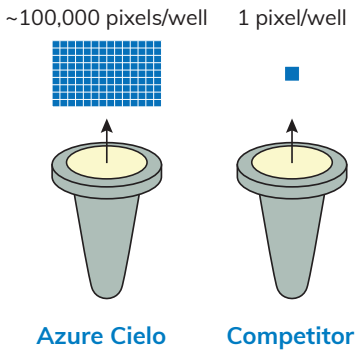

Figure 2. Azure Cielo acquires approximately 100,000 data points/well using total well detection technology. Competitive systems can acquire only 1 pixel/well compared to Azure Cielo Real-Time PCR systems that can capture approximately 100,000 pixels/well enabling accurate, reproducible and sensitive representation of fluorescent intensities from each qPCR well.

Materials and methods

cDNA preparation

10 ml cultures of Saccharomyces cerevisiae were grown at 30°C to OD600 0.5–0.8. Cells were washed in 1 ml cold water (809-115-CL, Wisent) and resuspended in buffer from the Aurum Total RNA mini kit (7326820, BioRad). Instead of using the suggested enzymatic lysis protocol, cells were lysed in buffer with 250 µl of glass beads using a FastPrep-24 (MP Biomedicals) for 2 x 45 seconds at 4 m/s, with a 2 min rest on ice in between cycles. The lysate was spun down, supernatant collected and processed using the Aurum kit according to manufacturer's instructions, including DNase treatment.

RNA was quantified by nanodrop and 10 µg of RNA was subjected to a further DNase I (M0303L, NEB) treatment for 10 minutes at 37°C in a total volume of 100 µl. 1 µl of 0.5 M EDTA was added and the RNA was purified and concentrated using RNA Clean & Concentrator-5 (with DNase treatment) (R1014, Zymo Research). RNA was quantified after DNase treatment and 3 µg of RNA was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (RR037B, Takara Bio), with the reaction volume scaled up accordingly.

Quantitative PCR

Quantitative PCR (qPCR) reactions were performed in a 96 well plate (AB0700W, Thermo Fisher) on an Azure Cielo 3 Real-Time PCR or a competitor qPCR instrument in a total volume of 20 µl using PerfeCTa® SYBR® Green Supermix (95054, Quantabio). The cDNA was diluted 1:10 before qPCR. For ACT1, on only the Cielo, the cDNA was diluted 1:20. The primer pairs used were:

Amplicon	Name	Sequence (5'-3')
actin	ACT1 F	AACGAATTGAGAGTTGCCCC
actin	ACT1 R	CAAGGACAAAACGGCTTGGATG
TEL06R	6R_F1	AAATGGCAAGGGTAAAAACCAG
TEL06R	6R_R1	TCGGATCACTACACACGGAAAT
6 Y' telomeres	oLK49[2]	GGCTTGGAGGAGACGTACATG
6 Y' telomeres	oLK50[2]	CTCGCTGTCACTCCTTACCCG

The final composition of each reaction was:

- 10 µl 2x PerfeCTa® SYBR® Green Supermix
- 4 µl primer pair (150 nM final concentration)
- 2 µl 1:10 dilution of cDNA (1:20 for ACT1 on Cielo only)
- 4 µl H_2O
- 20 µl

Plates were sealed with sealing tape and qPCR carried out according to the following protocol:

1. Initialization at 95°C for 2 min
2. Denaturation at 95°C for 15 sec
3. Annealing at 60°C for 30 sec followed by plate read
4. Extension at 72°C for 30 sec
    - Repeat steps 2–4 for a total of 40 cycles

Calculation of primer efficiency

qPCR reactions were performed using a series of four 4-fold dilutions of cDNA and a no template control. Each sample was assessed in duplicate. Efficiency was calculated by plotting Cq values vs dilution factor.

Results and discussion

The efficiencies of the high- and low-abundance qPCR reactions were very similar between the Cielo and the competitor machine (Table 1). However, the efficiency of the very-low abundance TEL06R reaction could not be determined for the competitor instrument because the Cq values were very late and compressed between cycles 35 and 38, resulting in a low value and impossible calculated efficiency greater than 200%.

For each of the three targets examined, the Cielo demonstrated significantly earlier Cq values indicating greater sensitivity (Table 2).

Amplicon	Cielo Efficiency (R^2)	Competitor Efficiency (R^2)
ACT1	90% (0.998)	90% (0.996)
Y' TERRA	106% (1.00)	107% (0.997)
TEL06R TERRA	96% (0.999)	Not determined

Table 1. Amplification efficiency observed with the Cielo and a competitor.

ACT1 is an essential gene encoding actin, expected to be highly expressed and very commonly used as a reference in gene expression studies.[3] Though the Cielo reactions contained half as much starting cDNA as the competitor reactions, the Cq value for the Cielo was earlier than for the competitor instrument (delta Cq = 0.65)(Table 2).

This difference in Cq values was greater for the lower-abundance targets. TERRA originating from the Y' subtelomeric element of six telomeres is a telomeric repeat-containing long noncoding RNA and is expected to be low abundance while TEL06R TERRA is a target originating from the subtelomere of TEL06R and expected to be very-low abundance (single copy). The Cq value for the Cielo was more than 2 cycles earlier for Y' TERRA and 4 cycles earlier for TEL06R TERRA than with the competitor instrument (Table 2). These results indicate the effect of increased sensitivity of the Cielo becomes more important as the amount of starting target decreases.

Amplicon	Cielo Cq, mean (st dev)	Competitor Cq, mean (st dev)	Cq Competitor – Cq Cielo
ACT1	16.75 (0.17)	17.40 (0.20)	0.65
Y' TERRA	25.55 (0.07)	27.80 (0.28)	2.25
TEL06R TERRA	30.20 (0.32)	34.22 (0.22)	4.02

Table 2. Cq values observed with the Cielo and a competitor.

Sensitivity is important to accuracy and reproducibility. As the number of PCR cycles increases, the potential for amplification of non-specific products or primer dimers also increases. This is particularly important when using non-specific fluorophores like SYBR green to detect PCR product. Therefore, the ability to detect amplification at earlier cycles increases the reliability of the data.

The Cielo with individual well illumination and detection is designed to minimize background noise and maximize sensitivity for the highest possible specificity and precision in qPCR reactions. See https://www.azurebiosystems.com/cielo-6/ to learn more about the Azure Cielo and how it can help you optimize your qPCR experiments

References

1. Bustin SA, Benes V, Garson JA, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin Chem.* 2009;55:4(611-622).
2. Graf M, Bonetti D, Lockhart A, et al. Telomere Length Determines TERRA and R-Loop Regulation through the Cell Cycle. *Cell.* 2017;170:1(72-85).
3. Teste MA, Duquenne M, Francois JM, Parrou JL. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in Saccharomyces cerevisiae. *BMC Mol Biol.* 2009;10:99.