Multiplex PCR on QuantStudio instruments: getting more data more efficiently

Introduction

Biology is very complex, with practically every component depending on every other component. For example, the transcriptome, which affects the proteome and metabolome, depends on the genome, which is also affected by the proteome and epigenome. The influences of tissue and environmental factors further complicate analyses. Therefore, in order to understand even the most basic aspect of a biological process, multiple factors have to be considered simultaneously.

qPCR analysis

The polymerase chain reaction (PCR), which amplifies specific sequences of DNA, has become an integral part of nearly every nucleic acid analysis technique. In some of these analyses, where determining the amount of a target in a sample is necessary, the basic PCR technique has been modified to enable quantification [1]. In most cases, quantitative PCR (qPCR) works by utilizing an oligonucleotide probe that is labeled with a fluorescent dye and quencher. This probe is designed to target a location between two PCR primers. During PCR amplification, the polymerase copies the sequence between the primers and degrades the bound probe, releasing the dye and increasing the fluorescence signal proportionally to the amount of target DNA [2].

qPCR is therefore a necessary tool for understanding biological systems. However, as the types of questions being asked become more complex, and the amount of sample available for analysis becomes smaller, researchers are running into the limitations of single-assay qPCR. For example, fine-needle biopsies [3] and liquid biopsies [4] are less-invasive methods for obtaining samples for oncology research. The amounts of DNA recovered from such collections are usually small, and the DNA is often fragmented. Since the sample often has to be divided into separate reactions for qPCR analysis of individual targets, the number of data points that can be set up can be small, limiting the complexity of the questions being asked.

Multiplex qPCR

To get around this limitation, researchers have been turning to multiplex analyses. In multiplex qPCR experiments, different nucleic acid targets in a single sample can be analyzed with qPCR assays containing probes labeled with different-colored fluorescent dyes. As an example, the β-actin gene might be analyzed with a blue probe (Applied Biosystems™ FAM™ dye), *GAPDH* with a green probe (Applied Biosystems™ VIC™ dye), *GUSB* with a yellow probe (Applied Biosystems™ ABY™ dye), and *KRAS* with a red probe (Applied Biosystems™ JUN™ dye) (Figure 1). The mixture of DNA in the sample with the four probes is then run in a single well or tube. This setup allows a researcher to get four times as much information from a single sample, since the sample is queried in a single reaction, instead of in four separate reactions. Multiplexing not only saves sample but also effort, since a single well or tube is used for the setup instead of four separate wells or tubes.

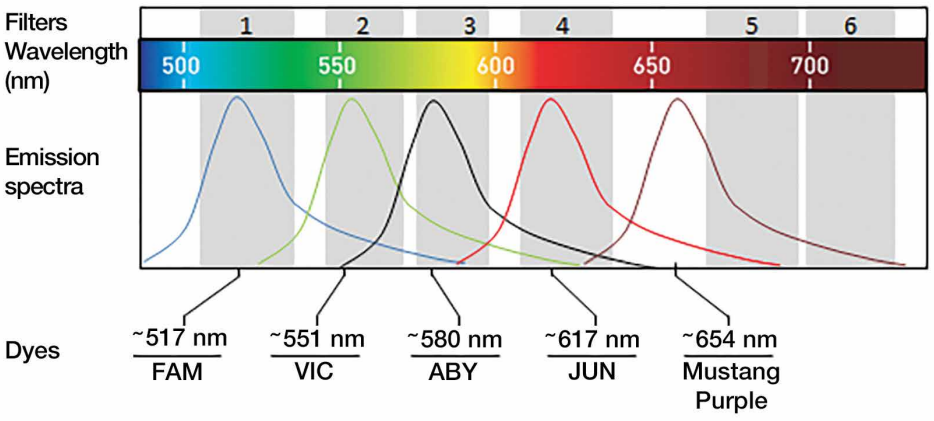

Figure 1. Fluorescence emission spectra of different dyes used for multiplex qPCR. Combinations of excitation and emission filters can be used to isolate each of the different colors, so there is minimal crosstalk between spectra. This facilitates the detection of multiple targets in a single reaction.

qPCR instrumentation

The latest Applied Biosystems™ QuantStudio™ qPCR instruments have been built to accommodate multiplex analysis with Applied Biosystems™ TaqMan™ Assays (Table 1). The latest QuantStudio instruments include the Applied Biosystems™ OptiFlex™ system, which improves well-to-well and instrument-to-instrument data accuracy. This system enables advanced multiplexing of multiple genes through decoupled excitation and emission filter channels (Figure 2). With the OptiFlex system, multiple fluorescent assays, including TaqMan Assays, can be analyzed within the same well.

Data quality

Although they have sophisticated multiplexing capabilities, these instruments preserve the high standards for data quality of the QuantStudio qPCR instruments. A dynamic range of over six orders of magnitude of target facilitates detection of broad changes between different types of samples and conditions (Figure 3). And even in a multiplex reaction, QuantStudio qPCR instruments can detect very small changes, as low as 1.5 fold, in target abundance (Figure 4). Detecting these small differences are crucial for understanding how gene expression changes in a biological system.

Multiplex qPCR applications

The ability to analyze multiple targets at one time has been useful for querying low-abundance samples and increasing efficiencies in research laboratories. For example, Jin et al. used duplex TaqMan qPCR assays to query methylation status in liquid biopsy research samples from colon cancers [5]. The analysis of long noncoding RNAs in thyroid cancers was performed using a multiplex reaction of four TaqMan qPCR assays [6]. To increase throughput and reduce costs, a multiplexed TaqMan qPCR assay was used to detect different species of *Anopheles* mosquitos [7]. Finally, Schwarz et al. developed a multiplex set of housekeeping gene probes to rapidly identify which would be most appropriate as a reference target in gene expression research studies [8].

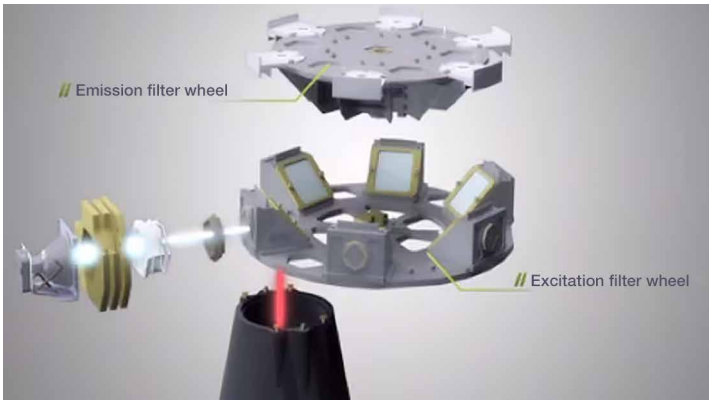

Figure 2. The OptiFlex system enables advanced multiplexing through decoupled excitation and emission filter channels.

Table 1. Fluorescence emission spectra of different dyes used for multiplex qPCR and detectable using the QuantStudio family of instruments.

Channel	1	2	3	4	5	6
Excitation filter	470 ± 15 nm	520 ± 10 nm	550 ± 10 nm	580 ± 10 nm	640 ± 10 nm	662 ± 10 nm
Emission filter	520 ± 15 nm	558 ± 12 nm	586 ± 10 nm	623 ± 14 nm	682 ± 14 nm	711 ± 12 nm
Dye examples	FAM, SYBR, SYTO 9, MeltDoctor, fluorescein	VIC, JOE, TET, HEX	TAMRA, NED, BODIPY TMR-X, ABY	ROX, Texas Red, JUN	LIZ, Cy5, Mustang Purple	Cy5.5, Alexa Fluor, fluorescein
QuantStudio 1 instrument	✓	✓		✓		
QuantStudio 3 instrument	✓	✓	✓	✓		
QuantStudio 6 Pro instrument	✓	✓	✓	✓	✓	
QuantStudio 5, 7 Pro, and 12K Flex instruments	✓	✓	✓	✓	✓	✓

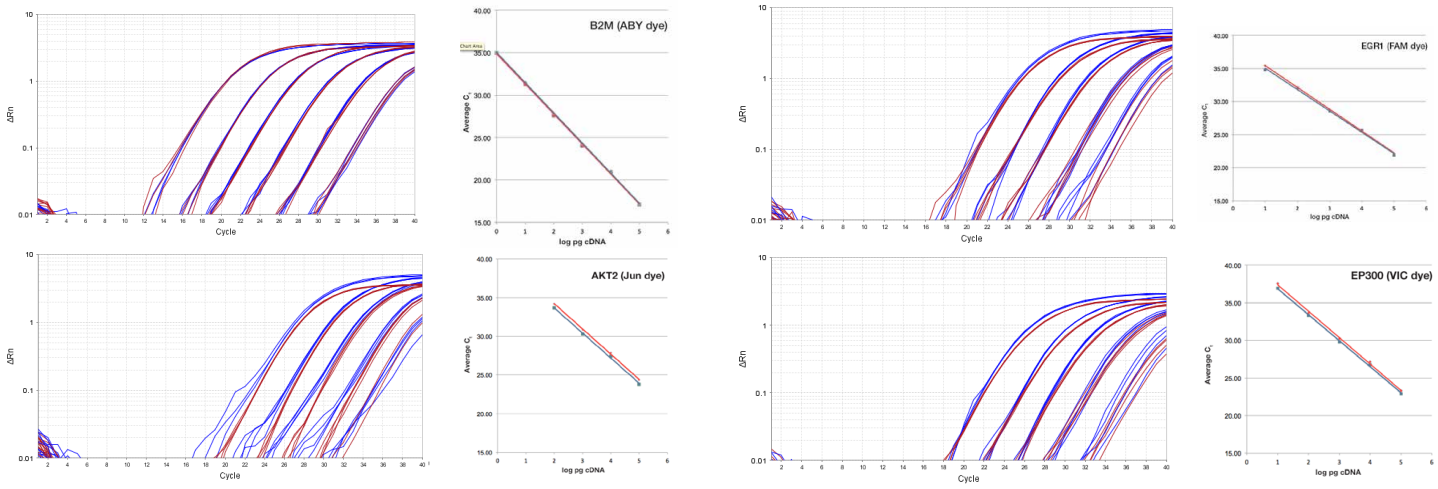

Figure 3. Comparison of results in singleplex and 4-plex reactions. Amplification was performed using a serial dilution of reference colon cDNA from 20,000 pg to 2 pg per 10 µL reaction. In all four amplification plots, blue data are from 4-plex reactions and red are from singleplex reactions. For more information, see our application note: **TaqMan multiplex PCR: Accurate, sensitive, and as efficient as traditional qPCR**.

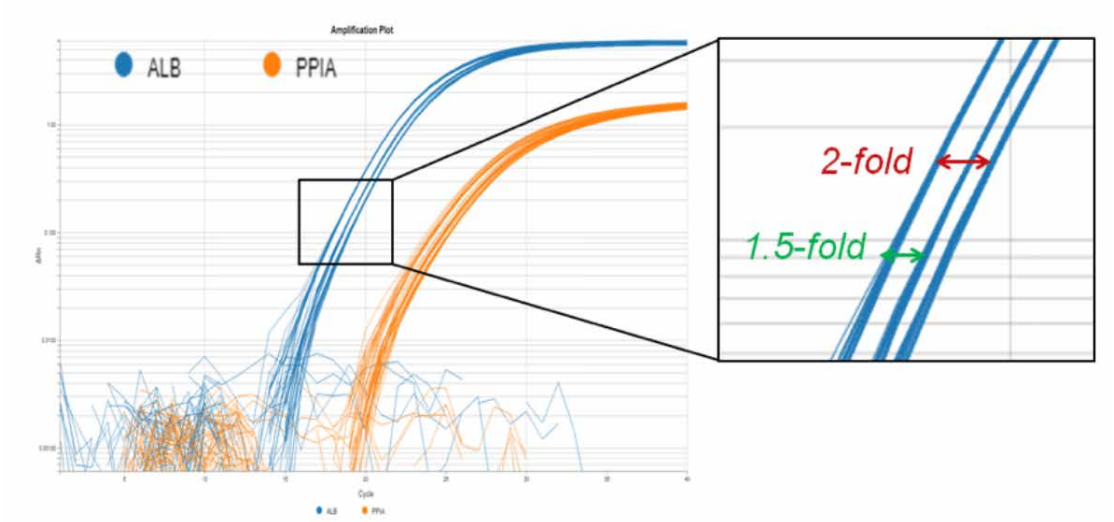

Figure 4. Detection of 1.5-fold change in transcript abundance in a duplex reaction. Total liver cDNA was titrated and analyzed with *ALB* and *PPIA* TaqMan Assays in a duplex reaction on an Applied Biosystems™ QuantStudio™ 7 Pro instrument. Note the discrimination between 2- and 1.5-fold transcript abundances in the inset. For more information, see our white paper: **Performance comparison of QuantStudio 7 Pro, QuantStudio 7 Flex, and 7900HT Real-Time PCR Systems**.

Conclusions

The current line of Applied Biosystems™ qPCR instruments builds on decades of innovations developed by our chemists and engineers. The OptiFlex system for multiplex PCR analysis, incorporated into our most modern instruments, is one innovation that helps bring increased control over your laboratory research workflows. Having more control over the number of targets analyzed in a single reaction helps instill confidence that your research is the best it can be.

To learn more about how multiplexing technology can help you with your research, **contact a sales representative** for a demo or a quote.

References

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