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QuantStudio 3 and 5 Real-Time PCR Systems

Intuitive benchtop instruments provide maximum efficiency and performance across a range of qPCR applications

In this application note, we show:

• Human and plant genotyping

• Pathogen detection and antibiotic resistance screening

• miRNA quantification and multiplex gene expression analysis

• Screening annealing temperatures for rare allele detection custom assays

• Optimizing high resolution melt (HRM) and SYBR™ Green conditions

The Applied Biosystems™ QuantStudio™ 3 and 5 Real-Time PCR Systems allow maximum efficiency with a high level of performance for multiple applications, including genotyping, miRNA and gene expression analysis, copy number determination, strain identification, presence/absence detection, and melt curve analysis.

Introduction

At the core of the QuantStudio 3 and 5 systems are Applied Biosystems™ VeriFlex™ and OptiFlex™ technologies. OptiFlex technology provides enhanced fluorescence detection enabling accurate and sensitive data analysis, while VeriFlex™ Blocks provide independent temperature zones for precise temperature control over your qPCR optimization. An interactive touch-screen interface and simplified Applied Biosystems™ QuantStudio™ Design and Analysis Software make it easy to get started and stay organized. The easy and intuitive interface allows running and editing of programs directly from the touch screen. For easier collaboration, the QuantStudio systems can upload data into the cloud to allow users to access and share data from any location with internet access.

Figure 1. QuantStudio 3 and 5 Real-Time PCR System conectivity: stand alone, desktop, or online. The QuantStudio 3 and 5 systems can connect by laptop to the QuantStudio Design and Analysis Software. The systems can also connect to the QuantStudio Design and Analysis Software on Thermo Fisher Cloud using any device with a web browser. The system may also be used as a stand-alone instrument, with data transfer by USB or Wi-Fi connection to the web browser–based software.

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QuantStudio Design and Analysis Software

The QuantStudio 3 and 5 systems can be accessed through the QuantStudio Design and Analysis Software either from a computer or from a web browser through an internet connection. The software is streamlined and easy to use with protocol templates for many applications. The web browser–based system configuration is compatible with PC or Mac™ computers. The web browser–based software allows for integration and analysis of multiple data sets and data types into one project. The software is compatible with most Applied Biosystems™ real-time PCR systems. Data sets can be quickly shared and large files can be sent securely around campus or around the world.

An example of data from the desktop software is shown by a maize genotyping experiment. Applied Biosystems™ Custom TaqMan™ SNP Genotyping Assays targeting biallelic SNPs were designed using the Applied Biosystems™ Custom TaqMan™ Assay Design Tool (**thermofisher.com/cadt**). Crude

lysates from maize seed chips were generated by the Applied Biosystems™ DNA Extract All Reagents Kit and used directly in qPCR by adding Applied Biosystems™ TaqMan™ GTXpress™ Master Mix. The standard genotyping experiment method was used on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System. The allelic discrimination plot created by the desktop software (Figure 2A) shows the trajectory of DNA samples by cycle (gray lines), allowing determination of the number of cycles that gives the best combination of allelic discrimination and signal intensity. This feature also helps identify genotypes of undetermined or ambiguous samples by showing the trajectory of the curve from a sample relative to those of other samples within genotype clusters. The genotype calls shown were made automatically by the desktop software. The cloud software has the added capability of displaying a series of allelic discrimination plots from multiple assays, providing a quick view of results within a project (Figure 2B).
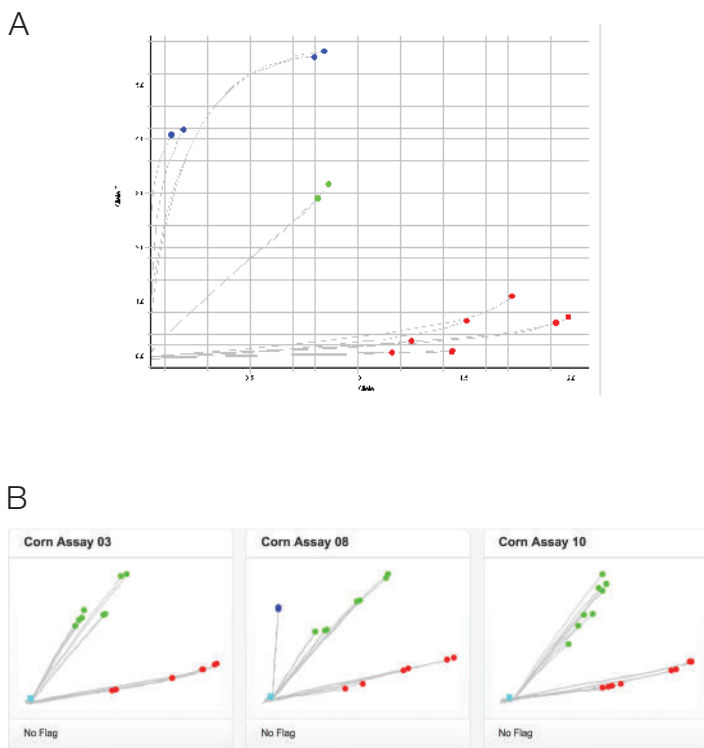
A

B

Figure 2. Maize genotyping with Custom TaqMan SNP Genotyping Assays. (A) Allelic discrimination plot created by the desktop software. **(B)** Series of allelic discrimination plots from the Thermo Fisher Cloud interface. Gray lines showing the path of each sample through cycle 50 were generated using real-time PCR data. Automated genotyping calls were generated by both software platforms.
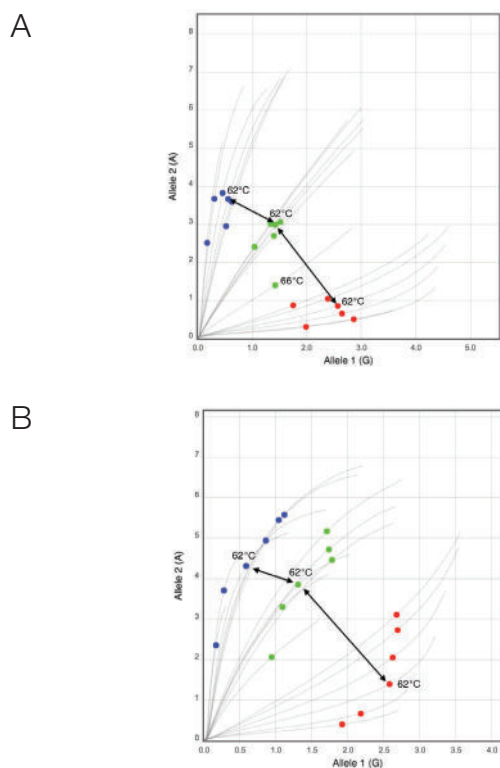
A

B

Figure 3. Screening annealing temperatures using VeriFlex zones. (A) A custom TaqMan SNP Genotyping Assay was tested using Applied Biosystems™ TaqMan™ Genotyping Master Mix and annealing temperatures ranging from 56 to 66°C. **(B)** A similar experiment using Applied Biosystems™ QuantStudio™ 3D Digital PCR Master Mix. In contrast to A, the signal and cluster separation are more strongly affected by temperature. Allelic discrimination between the non-mutant and mutant allele is best at 62°C based on the degree of cluster separation.

Optimizing probe hybridization for allelic discrimination using VeriFlex zones

The QuantStudio 3 and 5 systems with a 96-well configuration can be used to screen a range of annealing temperatures on a single plate to optimize experimental conditions. In Figure 3, results from different annealing temperatures are shown for a Custom TaqMan SNP Genotyping Assay designed to detect a rare mutation by digital PCR. This assay is known to be sensitive to annealing temperature based on results from Applied Biosystems™ QuantStudio™ 3D Digital PCR Chips where the assay shows lower specificity at 56°C and higher allelic discrimination at 62°C. The samples used for testing in these experiments are human genomic DNA homozygous for the non-mutant allele, a plasmid containing the rare

mutation, and a combination of genomic DNA and plasmid mixed at a 1:1 copy number ratio.

The assay performed well on the Applied Biosystems™ QuantStudio™ 5 system in an allelic discrimination experiment using TaqMan Genotyping Master Mix, showing slight annealing temperature effects on allelic discrimination at cycle 40 (Figure 3A). However, more significant annealing temperature effects were observed when the assay was run using QuantStudio 3D Digital PCR Master Mix (Figure 3B), showing that the assays behave differently in the two master mixes. This experiment demonstrates that the QuantStudio 5 system can be used to quickly screen optimal annealing temperatures for custom assays used in rare allele detection. Results from this screening may be
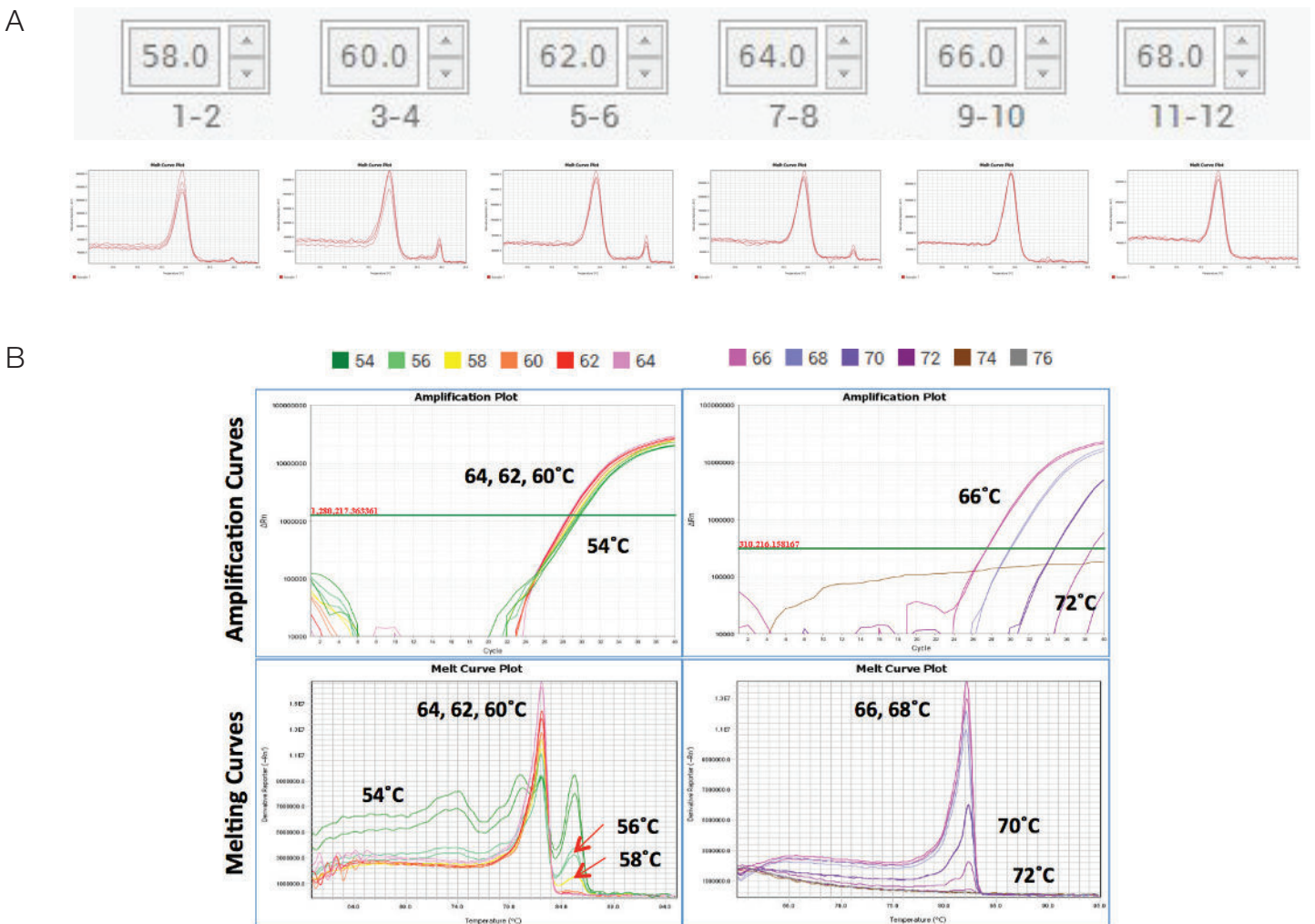

Figure 4. Annealing temperature optimization using VeriFlex zones. (A) Melt curves from SYBR Green primers tested with 58 to 68°C annealing temperature in Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix. A nonspecific product on the right side of the melt curve is absent at 66 and 68°C. **(B)** Primer annealing temperature data from 54 to 64°C (left) and 66 to 72°C (right). Nonspecific products are visible in melt curves on either side of the main peak from 54 to 58°C. A single peak is observed at temperatures ranging from 60 to 64°C. At even higher temperatures between 66 to 72°C (right), less signal is observed in both the amplification and melt plots.

beneficial when combined with further optimization directly on the QuantStudio 3D Digital PCR System.

Optimizing SYBR Green and HRM primer annealing temperatures using VeriFlex zones

SYBR Green assays provide certain advantages with regards to cost and design, but have a higher risk of nonspecific signal and require the addition of a melt stage to verify that only a single product was amplified after qPCR. Using an optimal annealing temperature can significantly reduce the amplification of nonspecific products. However, melt curve analysis at different temperatures is required to identify a temperature at which a specific amplicon is produced. The QuantStudio 3 and 5 systems can be used to screen a range of annealing temperatures on a single plate to identify an appropriate temperature for these assays. Figure 4A shows melt curve plots from SYBR Green qPCR primers tested at annealing temperatures ranging from 58 to 68°C in 2°C increments. At lower temperatures a second nonspecific peak is visible until the annealing temperature reaches 66°C, suggesting that the reactions should be run using an annealing temperature of 66°C or 68°C.

Applied Biosystems™ VeriFlex™ zones can also be used to optimize high resolution melt (HRM) PCR conditions. HRM analysis is used to detect genetic variations in nucleic acid sequences. Figure 4B shows melt curve plots for HRM primers tested from 54 to 72°C in two-degree increments. Annealing temperatures from 54 to 64°C were evaluated for primers using Applied Biosystems™ MeltDoctor™ HRM Master Mix (left graphs). Nonspecific products are visible in melt curves at lower temperatures ranging from 54 to 58°C. However, a single peak is observed at higher temperatures ranging from 60 to 64°C, demonstrating that annealing temperatures should be between 60 and 64°C. At even higher temperatures between 66 and 72°C (right graphs), less signal is observed based on the higher C_t values in the amplification plots and lower signal in the melt curves, demonstrating that this temperature range is too high. A single peak indicating the presence of a single amplicon is observed at or above 60°C, with decreasing peak height and higher C_t values above 66°C, indicating that a 60–66°C annealing temperature range is optimal.

Pathogenic strain identification

The QuantStudio 5 system can be used for pathogenic strain identification, and the instrument is able to automatically upload data to the cloud for online analysis or access from any location with an internet connection. This allows data to be shared with others for more efficient
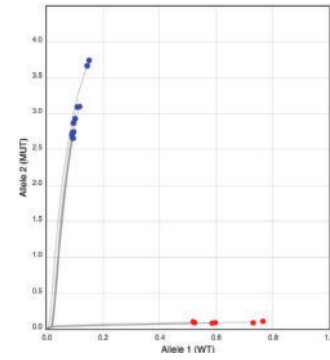

Figure 5. Allelic discrimination of *Mycobacterium tuberculosis* samples. Samples containing the wild type allele are shown in red, and samples containing a rifampin resistance allele are shown in blue.
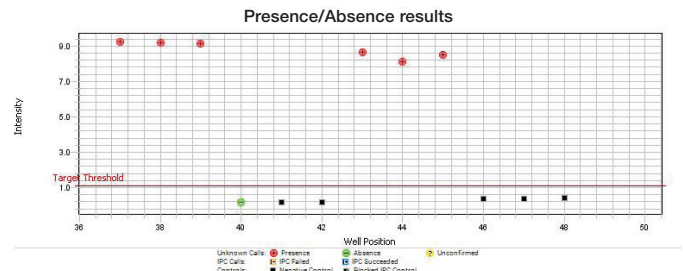

Figure 6. Rapid pathogen detection presence/absence study. Data showing detection of HSV-1 in samples from human swabs using the QuantStudio 5 Real-Time PCR System. Positive samples are shown in red above the threshold. A negative control is shown in green below the threshold. Negative samples appear in black.
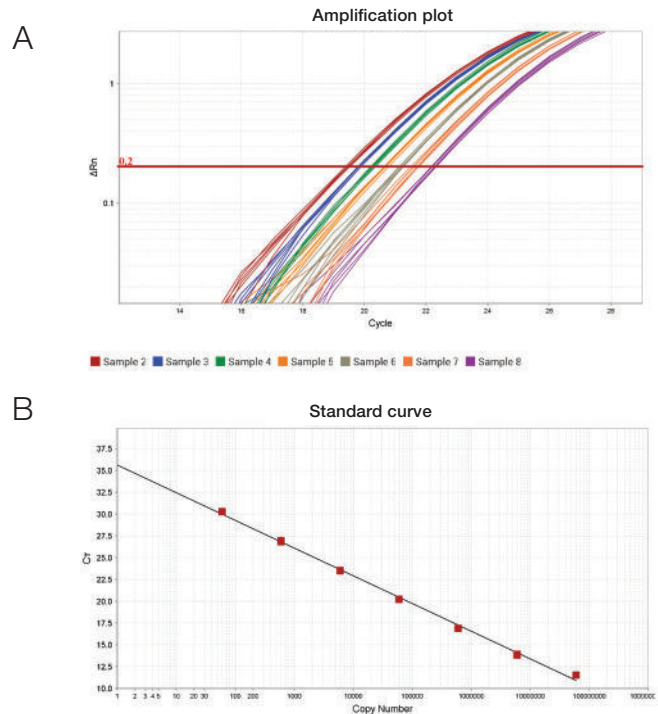

Figure 7. High precision and wide dynamic range measurements for miRNA analysis. (A) 1.5-fold dilution series of prostate cDNA analyzed with an Applied Biosystems™ TaqMan™ Advanced miRNA Assay targeting hsa-miR-16 (8 replicates, C_t SD < 0.2). **(B)** A 6-log linear dynamic range demonstrated on the QuantStudio 5 Real-Time PCR System and a TaqMan Advanced miRNA Assay targeting hsa-miR-378.

communication. Figure 5 shows results from an assay targeting the *rpoB* gene of *Mycobacterium tuberculosis*. The assay consists of FAM™ dye– and VIC™ dye–labeled probes and two primers. VIC dye is used to detect the wild type allele and FAM dye is used to detect a mutation associated with rifampin resistance (*rpoB* codon 531 TCG>TTG). Samples were tested in duplicate and the results were concordant between duplicates. Results clearly show discrimination between alleles, which is in agreement with known sequence information about each sample.

Pathogen detection using presence/absence experiments

The QuantStudio 3 and 5 systems can be used for rapid pathogen detection in DNA samples. In Figure 6 we show results from DNA purified from swab microflora samples tested for the presence or absence of herpes simplex virus 1 (HSV-1). Positive and negative controls were used to define the target threshold. A FAM dye–labeled TaqMan Assay was used to detect HSV-1 and a VIC dye–labeled Applied Biosystems™ TaqMan™ RNase P Assay was used as an internal positive control to detect human DNA known to be present in each sample. The Presence/Absence template on the instrument was run using Applied Biosystems™ TaqMan™ Fast Advanced Master Mix with a 40-minute run time.

The target threshold is shown in the graph as a solid horizontal line. Samples where HSV-1 was detected appear in red above the threshold, a negative control appears in green below the threshold, and negative samples appear in black below the threshold. Results show clear distinction between samples containing HSV-1 and samples that do not contain the pathogen (Figure 6). These results agree with known information about the samples.

High sensitivity and wide dynamic range measurements of miRNA expression

MicroRNAs (miRNAs) are short (~22 nucleotides), noncoding RNA molecules that posttranscriptionally regulate gene expression. They control diverse biological processes including cell proliferation, cell fate determination, and cell death. Applied Biosystems™ TaqMan™ miRNA Assays are considered to be the gold standard for quantifying miRNAs due to their unparalleled specificity and sensitivity. Here we demonstrate the quantification and precise discrimination of miRNAs using a TaqMan Advanced miRNA Assay and TaqMan Fast Advanced Master Mix on the QuantStudio 3 and 5 systems.

An amplification plot shows results from a 1.5-fold dilution series of cDNA (Figure 7A). Serum RNA from the prostate of a healthy donor was isolated using the Ambion™ *mir*Vana™ miRNA Isolation Kit. Total RNA (10 ng) was reverse-transcribed using the Applied Biosystems™ TaqMan™ Advanced miRNA cDNA Synthesis Kit. A 1.5-fold serial dilution of cDNA was then quantitatively analyzed with a TaqMan Advanced miRNA Assay targeting hsa-miR-16. The data represent 8 replicates with a C_t SD <0.2. The experiments were run using the Standard Curve Fast template on the QuantStudio 5 system.

TaqMan Advanced miRNA Assays run on the QuantStudio 5 system can provide up to a 6-log dynamic range (Figure 7B). Sequential 10-fold dilutions of synthetic miRNA target ranging from 60 to 600 million copies were reverse-transcribed using the TaqMan Advanced miRNA cDNA Synthesis Kit. The resulting cDNA was used in quadruplicate qPCR reactions using a TaqMan Advanced miRNA Assay for hsa-miR-378. Reactions containing as few as 60 copies produced sufficient signal for detection (C_t ~32.5). A standard curve of synthetic hsa-miR-279 amplification shows the linearity of the dynamic range. Results demonstrate the ability of the instruments to detect 1.5-fold differences in target levels in biological samples over 6-log dynamic range.

Multiplex gene expression analysis of a brain tumor cell line

Total RNA from SW1783 (HTB-13™) brain tumor cell line, which is known to harbor mutations in the *TP53* gene, were analyzed and compared to total RNA from normal brain.

Table 1. Multiplex dye configuration.

Gene symbol	5´ Dye
EGFR	FAM
MYC	VIC
B2M	ABY
BAX	JUN

To identify candidate genes that are differentially expressed in these samples, Applied Biosystems™ TaqMan™ Gene Expression Assays were designed that included QSY™ quencher, non-MGB probes. Assays for candidate genes in addition to a *B2M* assay (used as the endogenous control) were synthesized. Each assay was labeled with a different dye at the 5´ end and contained a QSY quencher at the 3´ end (Table 1).

Primer–probe mixes were formulated at 20X with 300 nM forward primer, 300 nM reverse primer, and
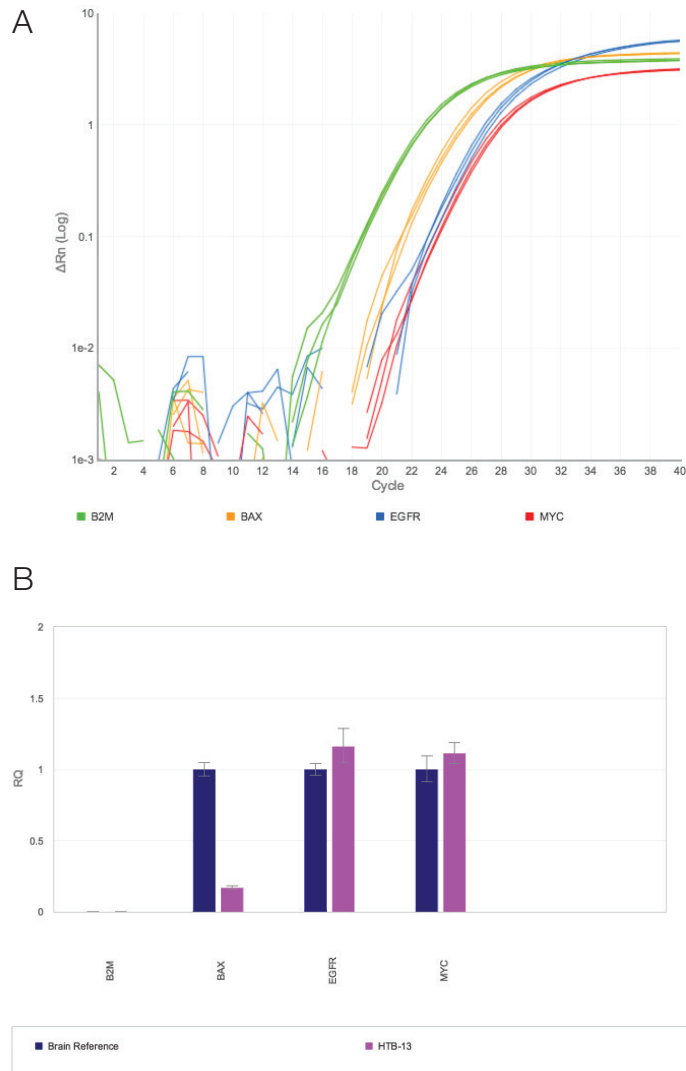
Figure 8. Multiplex gene expression analysis of a tumor cell line.
(A) Multiplex amplification plot showing data from brain cDNA used as a target. **(B)** The *B2M* gene was used as an endogenous control to show relative gene expression levels between normal brain tissue and SW1783 (HTB-13) brain tumor cell line with targets known to be down- and up-regulated based on the p53 mutation p.R273H.

References

1. Zacharatos PV, Gorgoulis VG, Kotsinas A et al. (1999) Modulation of wild-type p53 activity by mutant p53 R273H depends on the p53 responsive element (p53RE). A comparative study between the p53REs of the MDM2, WAFI/Cip1 and Bax genes in the lung cancer environment. WAFI/Cip1 = WAF1/Cip1. *Anticancer Res.* 19(1A):579–587.

2. Ludes-Meyers JH, Subler MA, Shivakumar CV et al. (1996) Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. *Mol Cell Biol* 16(11):6009–6019.

3. Frazier MW, He X, Wang J et al. (1998) Activation of c-*myc* gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain. *Mol Cell Biol* 18(7):3735–3743.

250 nM probe. Both RNA samples were converted to cDNA using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. The samples were then subjected to 14 rounds of preamplification using Applied Biosystems™ TaqMan™ PreAmp™ Master Mix to allow analysis of less than 1 ng of cell line DNA. The target assays (diluted 200 fold) were also used in the preamplification reaction. The multiplex relative quantification assays were run using Applied Biosystems™ TaqMan™ Multiplex Master Mix and the Standard Curve Fast template on the QuantStudio 5 system.

The average C_t for each assay was used to determine the ΔC_t between the target gene and *B2M* control gene to normalize for RNA input amount. Finally, to measure the fold change difference between the SW1783 RNA and reference brain RNA, the $\Delta\Delta C_t$ was calculated from the ΔC_t of the sample minus the ΔC_t of the reference for each assay (Figure 8). *BAX* expression was down-regulated by 5.1-fold, in agreement with previous results showing down-regulation as a result of the p.R273H mutation in p53 [1]. *EGFR* is known to be slightly up-regulated by the p.R273H mutation [2], and here a 1.3-fold up-regulation is observed between tumor cell line and normal tissue. *MYC* is known to be positively affected by mutations in p35 [3]. Here we observe a 1.26-fold increase in expression between tumor cell line and normal tissue. Assays run in singleplex showed results consistent with the multiplex reactions.

Summary

The QuantStudio 3 and 5 systems can be used for a wide range of applications including genotyping, miRNA and gene expression analysis, presence/absence detection, strain identification, and melt curve analysis. The instruments permit multiplex experiments for all of these applications. In addition, VeriFlex zones allow evaluation of up to 6 different temperature settings on one plate for rapid assay optimization. Multiple instrument features and streamlined software give researchers high performance and control over a variety of instrument parameters to obtain high-quality data with a simple workflow, and cloud connectivity allows convenient and rapid access to data and efficient communication of results.

Take a tour of the system at
thermofisher.com/quantstudio3-5

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