qPCR

Overcoming multiplexing challenges in molecular diagnostics and biopharmaceuticals: the power of analytically evaluated reagents

Introduction

Assay developers in biopharma and research labs strive to save time, reduce costs, and ensure accurate and reproducible results. In quantitative PCR (qPCR) assays, multiplexing—the simultaneous detection of multiple targets per reaction with a single sample input—offers a solution to achieve these goals. However, multiplexing, while maintaining accuracy and efficiency, as well as retaining performance at scale-up, poses significant hurdles. In this application note, we explore the challenges faced by assay developers in multiplexing with qPCR, and present data on validated reagents that enable assay developers to design their multiplex assay for up to 6 targets.

Multiplexing in qPCR requires careful consideration of various factors, such as assay design, reagent selection, and workflow optimization. Assay developers often encounter difficulties in achieving accurate and reliable results, particularly when using on-hand reagents for higher-order multiplexing. Time-consuming steps and challenges associated with assay design further compound the complexity in multiplexing experiments. Extended on-bench time may exacerbate unwanted interactions in a multiplex qPCR assay, causing inaccurate results. Therefore, benchtop-stable master mixes are often required to address needs for automation (see **our recent white paper**).

To address these pain points and empower assay developers with enhanced multiplexing capabilities, Applied Biosystems™ master mixes and probes have been specifically designed for multiplexing applications. These products, validated on Applied Biosystems™ QuantStudio™ PCR systems, support superior performance, accuracy, and sensitivity. Additionally, we offer an ecosystem of advantages, including comprehensive assay design tools and dedicated support, to assist assay developers in overcoming the challenges associated with multiplexing in qPCR assays.

Challenges and benefits of multiplexing

In the dynamic fields of research in biotechnology and biopharmaceuticals, multiplexing in qPCR has emerged as a crucial technique that allows simultaneous analysis of multiple targets in applications such as drug development, gene and cell therapy, and microbial detection. With multiplexing, the amounts of sample and reagents needed are greatly reduced, saving workflow time and cost. Multiplexing also increases the throughput and reduces variability while facilitating comparison of different targets within the same reaction.

Despite its numerous advantages, multiplexing in qPCR brings its own set of challenges that can affect the accuracy and reliability of results. Multiplexing assays are complex by nature. With the increased number of primers and probes necessary to amplify different targets, the chance of unwanted interaction between them, the target sequence, and amplicons generated in each PCR cycle increases. These challenges can compromise the accuracy and reproducibility of the results, limiting the potential of multiplexing.

Dynamic range and consistency of TaqPath DuraPlex 1-Step RT-qPCR Master Mix in higher-order multiplexing

Despite the challenges with multiplexing, there are ways to minimize the roadblocks to its success such as starting with reagents that have been specifically validated for multiplexing. Validated reagents developed for multiplexing undergo rigorous performance testing with multiplexed assays, providing confidence in their ability to facilitate accurate and reproducible results. Applied Biosystems™ TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix is developed, optimized, and validated for multiplexing up to 6 targets in a single reaction. Because targets in a multiplexed reaction may be present at varying levels in the sample, TaqPath DuraPlex master mix has a wide dynamic range that can help ensure the accurate detection of all targets in a multiplexed reaction.

In a 6-plex reaction, TaqPath DuraPlex master mix was able to amplify all 9 dilution points in a serial dilution series with RNA input of 10-fold difference between each dilution (Figure 1). Importantly, independently manufactured lots of TaqPath DuraPlex master mix tested with multiplexed reactions showed consistent performance (Figure 2), helping to ensure that potential experimental variability is not caused by using reagents that have not been validated for multiplexing applications.

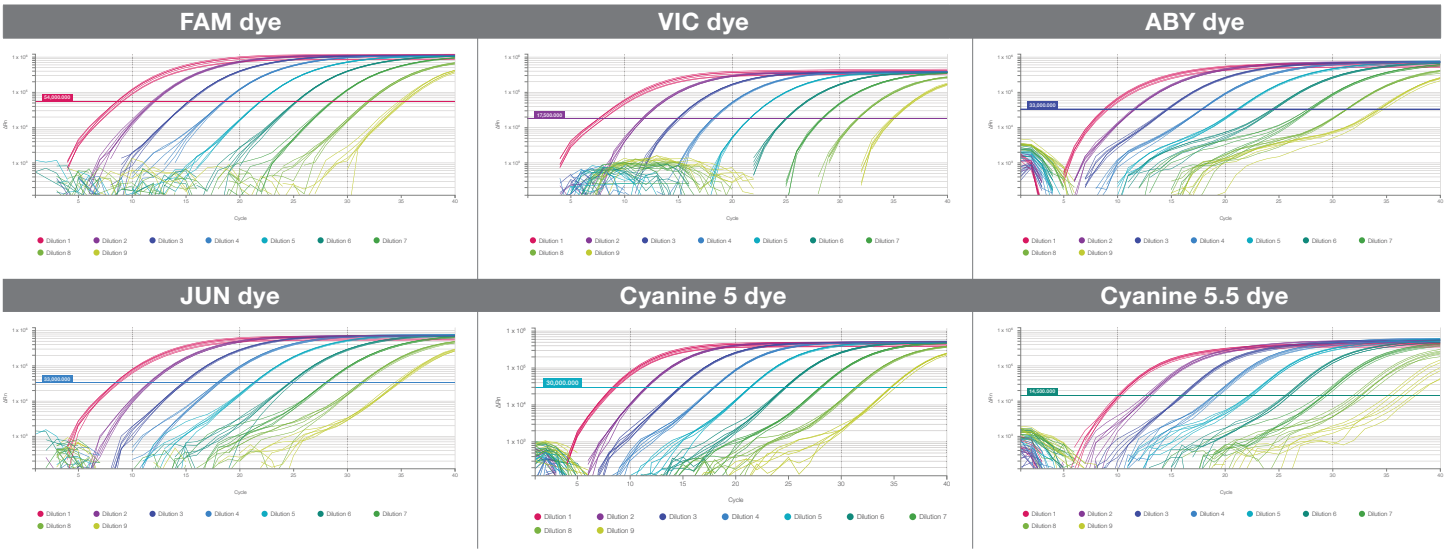

Figure 1. A master mix built for multiplexing capability has a wide dynamic range. Shown here is Applied Biosystems™ TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix, no ROX™ amplifying 6 targets in a single reaction across 8 logarithmic units of RNA input. Dyes used with the targets are Applied Biosystems™ FAM™, VIC™, ABY™, and JUN™ dyes, and cyanine 5 and cyanine 5.5 dyes.

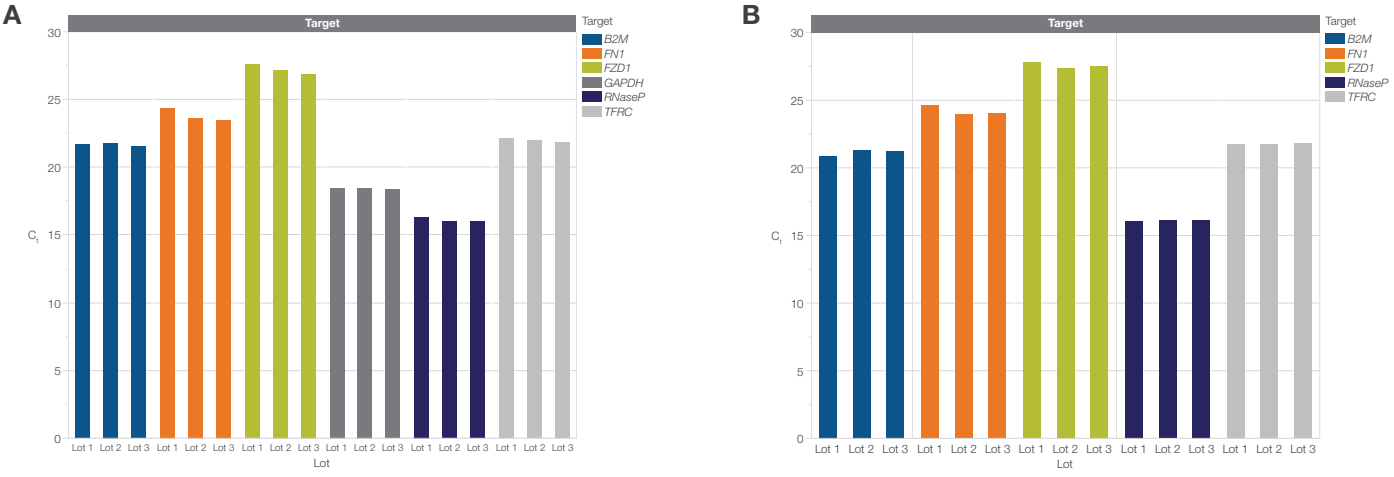

Figure 2. Different lots of TaqPath DuraPlex 1-Step RT-qPCR Master Mix show consistent performance in multiplexed RT-qPCR assays. **(A)** TaqPath DuraPlex 1-Step RT-qPCR Master Mix with no passive reference was tested in a multiplex assay with 6 human gene expression targets. **(B)** TaqPath DuraPlex 1-Step RT-qPCR Master Mix with ROX was tested with a multiplex assay using 5 human gene expression targets.

Impact of master mix on amplification efficiency in multiplexed reactions

Researchers often use reagents that are available on-hand for developing new assays, without considering the importance of whether that reagent is equipped to perform higher-order multiplexing in RT-qPCR. In a study comparing 1-step RT-qPCR master mixes from various suppliers, TaqPath DuraPlex 1-Step RT-qPCR Master Mix showed more consistent amplification in a multiplex assay across a broader dynamic range (Figure 3). Supplier 1's product had comparatively less consistent amplification, indicated by the 95% confidence areas shaded in gray. Importantly, supplier 2's product, which was advertised for 3 or more targets for multiplexing, struggled to amplify all dilution points in the same multiplex assay, and exhibited a loss of sensitivity compared to the other two master mixes. Altogether, these data demonstrate the need for using a master mix validated for higher-order multiplexing to achieve accurate and reproducible results.

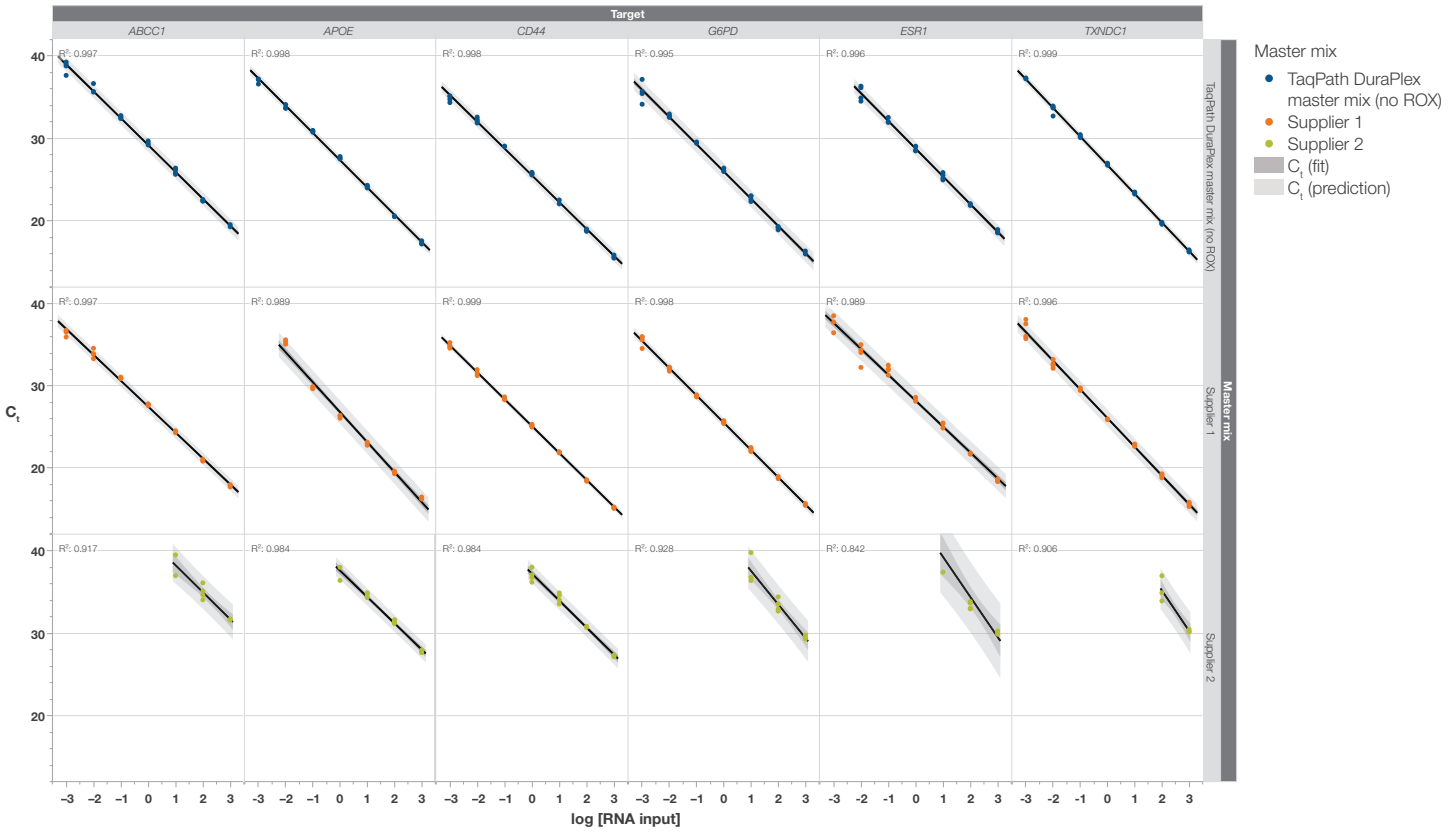

Figure 3. Choosing a master mix validated for higher-order multiplexing impacts the sensitivity and reliability of a multiplexed RT-qPCR experiment. Three different on-market 1-step RT-qPCR master mixes advertised for multiplexing were used in this study. TaqPath DuraPlex 1-Step RT-qPCR Master Mix (no ROX) is validated for 6 targets in a single reaction. The product from supplier 1 has multiplexing capability for up to 5 targets. The product from supplier 2 is advertised for 3 or more targets in a single reaction. RNA input (log scale) is graphed against C_t values to obtain R^2 values for each target. Targets and their associated dyes: *ABCC1* = FAM dye, *APOE* = VIC dye, *CD44* = ABY dye, *G6PD* = JUN dye, *ESR1* = cyanine 5, *TXNDC1* = cyanine 5.5.

In multiplexed reactions, targets may be present at varying abundance, and amplification of the low-abundance target may be suppressed in the presence of a high-abundance target. In a 5-plex reaction, TaqPath DuraPlex master mix was used to amplify the internal positive control (IPC) Applied Biosystems™ VetMAX™ Xeno™ Internal Positive Control RNA at a fixed input of 10,000 copies per reaction and 4 other gene expression targets in a serial dilution input of 1,000 ng to 0.001 ng of human RNA. The detection of the fixed-input target remained consistent across both high- and low-input dilutions of human RNA, showing that amplification from variable input concentrations is achievable (Figure 4A). In the same study, a single input concentration was run with single-target reactions for each of the 5 targets and compared to the performance of the corresponding targets in the multiplexed reaction. With TaqPath DuraPlex master mix, the scale-up from running single-target reactions to a 5-plex reaction showed no change in performance, demonstrating that no further optimization needed to be made for this multiplex reaction to produce accurate results in higher-order multiplexing (Figure 4B).

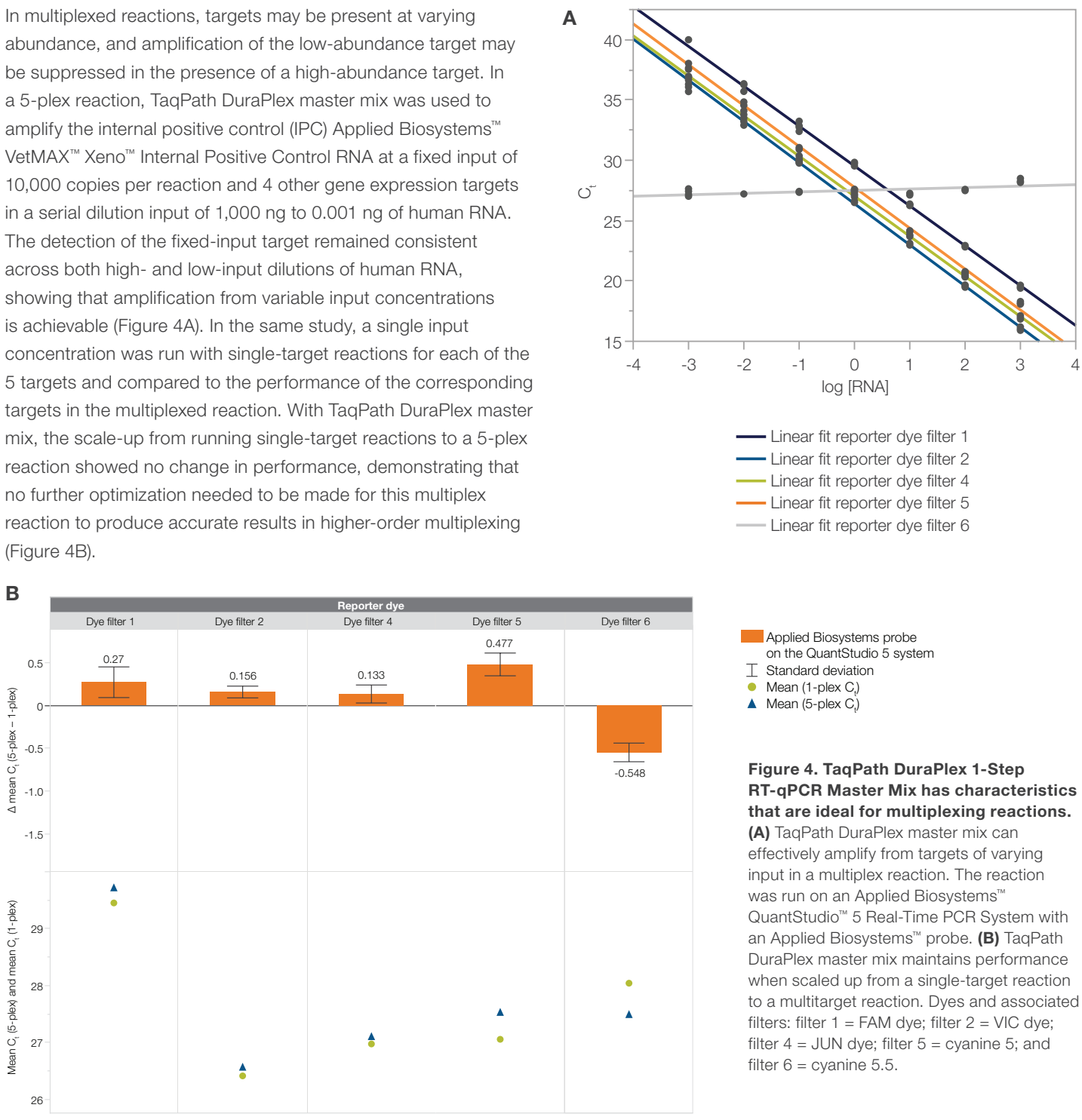

Figure 4. TaqPath DuraPlex 1-Step RT-qPCR Master Mix has characteristics that are ideal for multiplexing reactions. **(A)** TaqPath DuraPlex master mix can effectively amplify from targets of varying input in a multiplex reaction. The reaction was run on an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System with an Applied Biosystems™ probe. **(B)** TaqPath DuraPlex master mix maintains performance when scaled up from a single-target reaction to a multitarget reaction. Dyes and associated filters: filter 1 = FAM dye; filter 2 = VIC dye; filter 4 = JUN dye; filter 5 = cyanine 5; and filter 6 = cyanine 5.5.

Refer to **Applied Biosystems™ TaqMan™ QSY™ and QSY™2 probes** for more information on the probes used in the experiments (**link to probes section**).

The effects of probe type on multiplexing success: TaqMan QSY and QSY2 probes

When it comes to multiplexing, a common concern is performance change upon scaling up from a single target to multiple targets in one reaction. This can occur due to interactions between primers and probes for the different targets as well as their interactions with the template sequence. Aside from the master mix, the types of probes used can also impact the performance of a multiplexed reaction. The quencher-based TaqMan QSY2 probes are used with cyanine 5 and cyanine 5.5 reporter dyes, enabling researchers to multiplex up to 6 targets in combination with existing FAM, VIC, ABY, and JUN dyes. TaqMan QSY and QSY2 probes are designed for optimal performance in multiplexed studies, with better sensitivity and scale-up performance retention than other suppliers' products, as seen in our recent flyer.

TaqMan QSY2 probes perform consistently across different RT-qPCR instruments

Multiplexing can result in loss of sensitivity due to competitive inhibition between the different targets. This can be exacerbated with template inputs of varying concentrations or when the expression levels of the targets are drastically different. To examine this scenario, a VetMAX Xeno Internal Positive Control RNA was used at 10,000 copies per reaction with a dilution series of human RNA from 1,000 ng per reaction to 0.001 ng per reaction. In a 5-plex assay, TaqMan QSY2 probes were tested against probes from another supplier. TaqMan QSY2 probes showed earlier detection and higher signal (ΔRn) compared to the other supplier's probes (Figure 5). These differences were further analyzed on different real-time PCR instruments to address the concern of whether differences observed are due to calibration differences between the instruments, but the earlier C_t for TaqMan QSY2 probes was evident on both platforms (Figure 6).

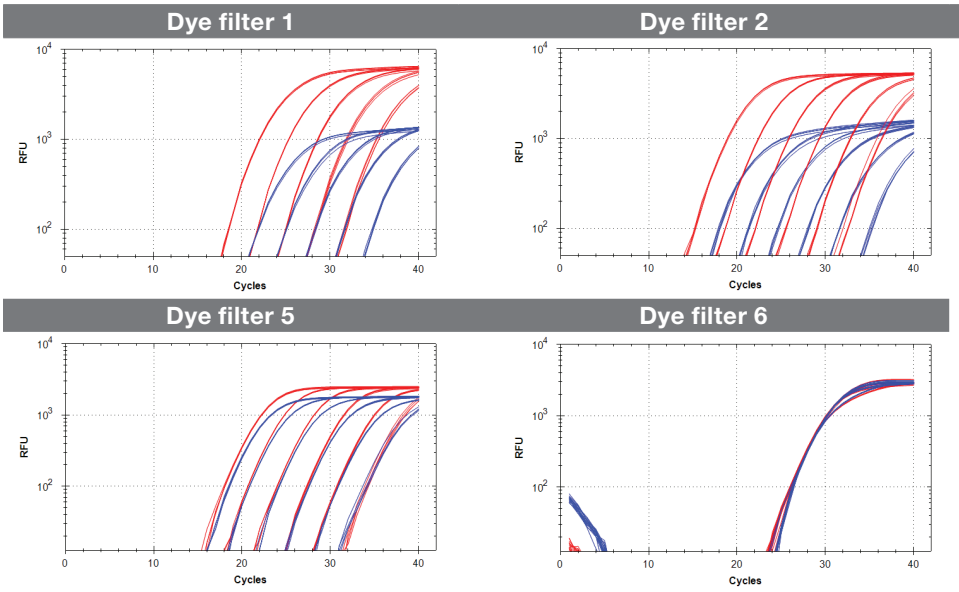

Figure 5. Performance of TaqMan QSY2 probes compared to another supplier's probes using a 5-plex reaction on the BioRad™ CFX™ Opus 96 Real-Time PCR System. The different amplification curves of the same color represent different concentrations in a serial dilution. Red amplification curves are those using TaqMan QSY2 probes. Blue amplification curves are those using the other supplier's probes. Filter 1 = FAM dye, filter 2 = VIC or HEX dye, filter 4 = JUN or ROX dye, filter 5 = cyanine 5 or Invitrogen™ Cy® 5 dye, filter 6 = cyanine 5.5 or Invitrogen™ Cy® 5.5 dye.

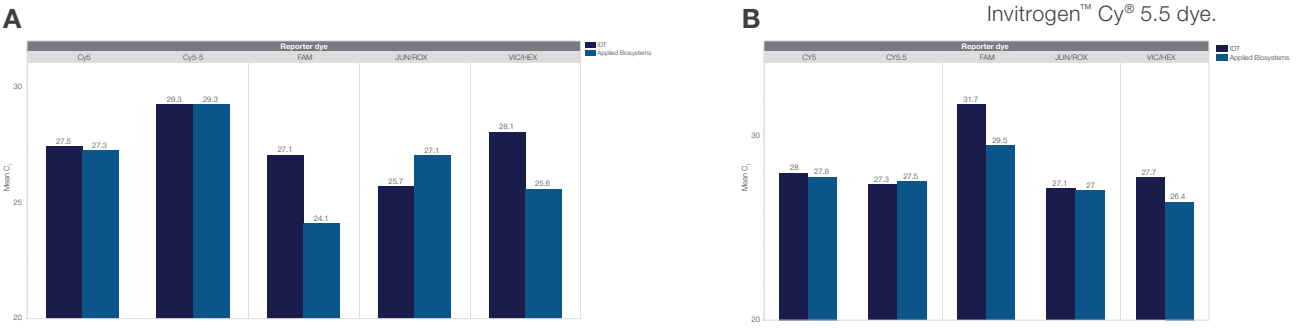

Figure 6. The TaqMan QSY2 probes provide better detection compared to another supplier's probes in a multiplexed reaction, regardless of instrument used. (A) The multiplexed reaction was run on a CFX Opus 96 instrument with IDT and Applied Biosystems probes. **(B)** The multiplexed reaction was run on a QuantStudio 5 instrument with IDT and Applied Biosystems probes.

With scale-up from a single-target reaction to multiplex reactions, there is often loss of performance due to interactions between different targets, their templates, and primers and probes. When comparing performance differences between simplex reactions to a 5-plex reaction with TaqMan QSY2 probes vs. probes from another supplier, TaqMan QSY2 probes showed less change in performance between the simplex FAM target reaction compared to the multiplexed reactions. This performance difference was not demonstrated to be influenced by the qPCR instrument used (Figure 7).

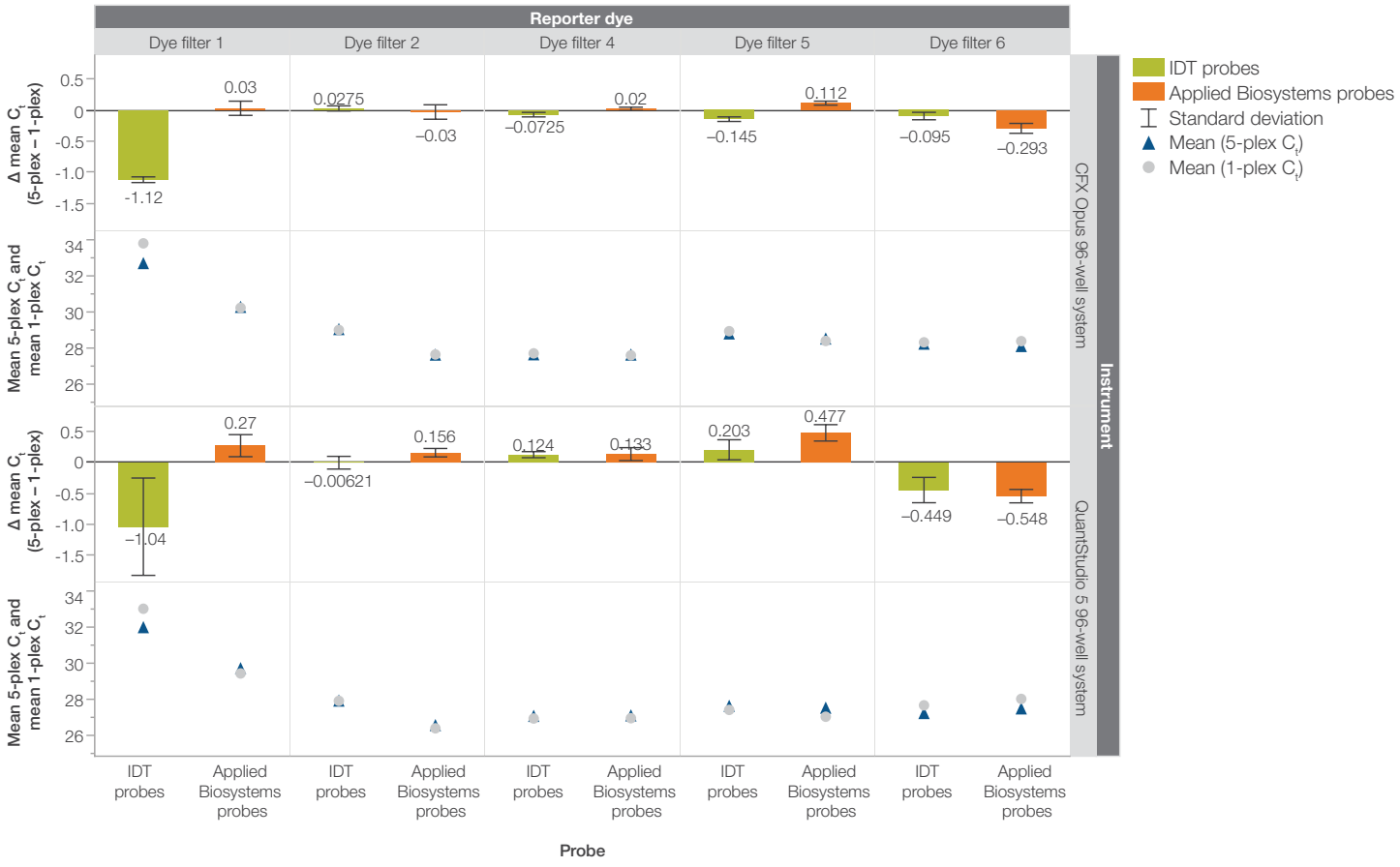

Figure 7. Comparison of scale-up performance change between probes offered by IDT and Thermo Fisher Scientific (Applied Biosystems). Top bars show data from the reactions run on the BioRad CFX Opus 96 Real-Time PCR System. Bottom bars show data from the reactions run on the QuantStudio 5 Real-Time PCR System**.** Filters and their associated dyes: filter 1 = FAM dye, filter 2 = VIC or HEX dye, filter 4 = JUN or ROX dye, filter 5 = cyanine 5 or Cy5, filter 6 = cyanine 5.5 or Cy5.5.

Refer to **TaqPath DuraPlex 1-Step RT-qPCR Master Mix** for more information on the master mix used in the experiments (**link to master mix section**).

Conclusion

The Applied Biosystems probes and master mixes offer a powerful solution for assay developers in biopharma and research labs seeking to optimize time, cost, and accuracy in their multiplexing experiments. By using validated reagents for multiplexing, one can help reduce the risks of having unreliable results due to a change in performance with the increased number of targets in a single reaction. By maximizing the number of targets per sample with multiplexing, researchers can reduce the time needed to develop multiplexed in-house assays while retaining excellent assay performance. TaqPath DuraPlex 1-Step RT-qPCR Master Mix is validated for up to 6 targets in a single reaction, with demonstrated lot-to-lot consistency in multiplexed reactions. Its wide dynamic range helps support the amplification of targets that have variable starting concentrations. Furthermore, its multiplexing capability helps retain performance when scaling up from a single reaction to multiplexed reactions.

Probe types also impact multiplexing performance, with the TaqMan QSY2 probes showing earlier detection and better multiplexing performance compared to probes from another supplier. The TaqMan QSY2 probes also help support the retention of performance with multiplexing scale-up. By embracing the benefits of multiplexing with validated probes and master mixes, assay developers can aim to elevate their research efficiency and accelerate scientific discoveries in a cost-effective manner.

Learn more at **thermofisher.com/qpcr-multiplex**