Application Note

Advancing higher-order multiplex PCR: Overcoming the limitations of qPCR with QIAcuity digital PCR

Introduction

Highly multiplexed PCR is a powerful technique that enables the detection of numerous targets within a single PCR reaction. By combining multiple PCR assays, researchers can maximize sample use, save time and minimize reagent consumption, thereby accelerating the process of obtaining deeper biological insights. This method has broad applications, from translational research to pathogen detection.

As the number of targets per reaction increases, so do the associated payoffs. Consequently, there are strong incentives for pushing multiplex PCR to its limits. However, multiplex PCR comes with two main challenges. First, as the number of amplicons rises, interactions between primers (for example, primer-dimer formations) and competition for reagents can lead to inconsistent or suboptimal amplification efficiency of certain targets. Second, optical crosstalk between neighboring channels can skew results, leading to inaccurate quantification.

qPCR is especially sensitive to shortcomings of multiplexing

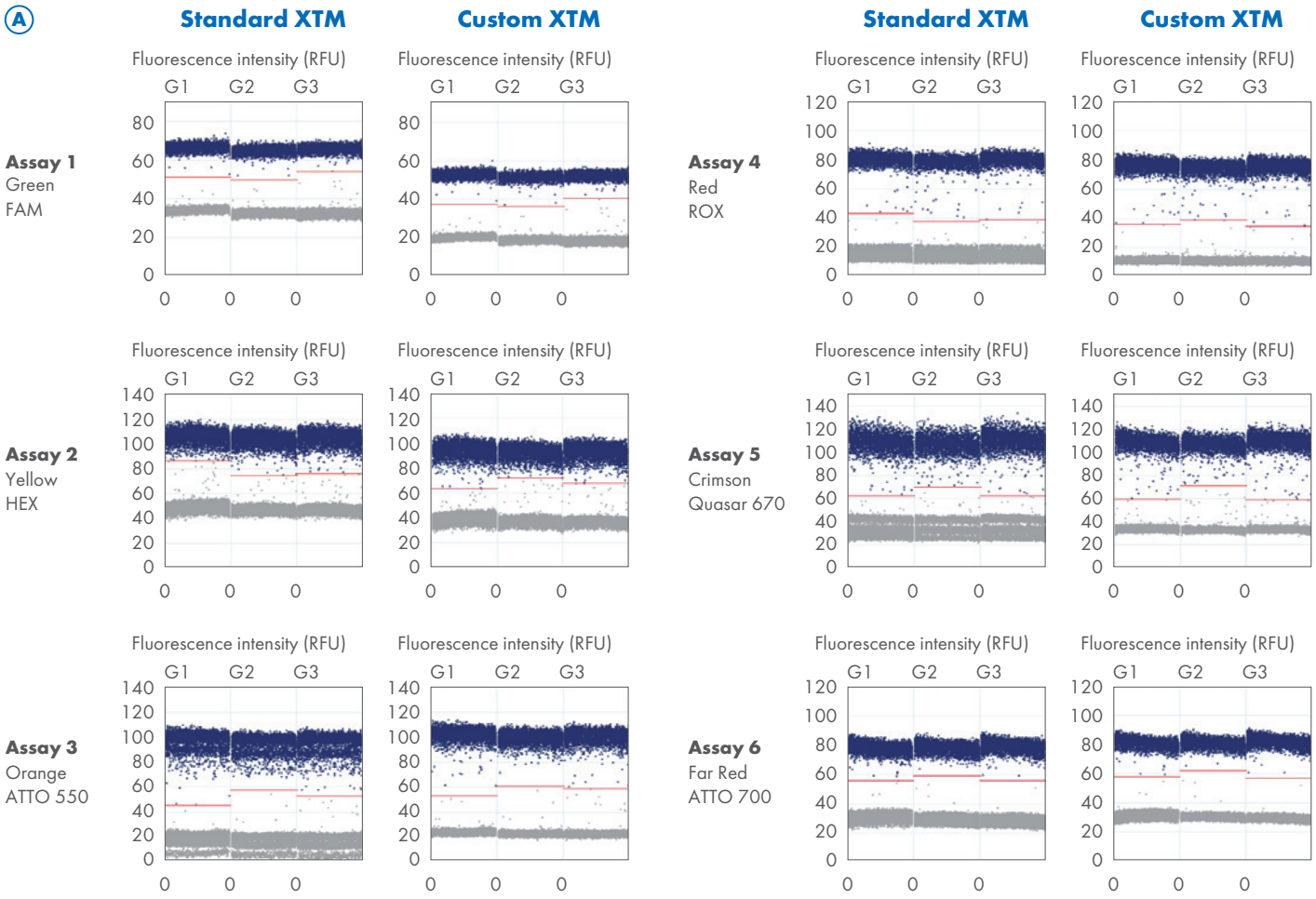
Multiplexed real-time quantitative PCR (qPCR) is a popular choice for quantifying multiple DNA or RNA targets in a single reaction. However, it is particularly vulnerable to the technical challenges associated with high multiplexing. Because qPCR relies on amplification efficiency for quantification, any shifts in PCR robustness stemming from the interractions between multiple amplicons will manifest as inaccurate underquantification and decreased sensitivity. This is of particular concern in highly multiplexed reactions. Conversely, crosstalk between assays in neighboring channels may artificially boost the quantification of certain targets. Therefore, qPCR users must tediously optimize and verify their multiplex setups in order to trust their results.

dPCR is an ideal option for higher-order multiplexing

The QIAcuity® digital PCR (dPCR) platform addresses and overcomes the challenges of high-multiplex PCR at the theoretical, software and chemistry levels. Unlike qPCR, which quantifies targets based on amplification curves, QIAcuity dPCR reactions are partitioned into thousands of individual reactions. By counting the number of positive partitions, QIAcuity dPCR delivers absolute quantification of DNA or RNA targets that is independent of PCR efficiency. Reaction partitioning also significantly reduces competition between targets, ensuring that even low-abundance sequences are accurately detected and quantified. Therefore, the fundamental approach of QIAcuity dPCR eliminates key sources of variability that are problematic for multiplexed qPCR.

At the chemistry level, the newly developed QIAcuity High Multiplex Probe PCR Kit uses a novel passive reference dye and specifically tailored PCR chemistry to let users conveniently perform higher-order multiplexing on existing QIAcuity hardware. An optimized reaction buffer and the highest concentration of DNA polymerase in any QIAcuity mix ensure optimal results for even the most challenging multiplex setups. ▷

Lastly, the newly released QIAcuity Suite 3.0 addresses concerns of unwanted optical crosstalk. In version 3.0, a refined default crosstalk compensation is automatically applied to reactions targeting up to six targets in the Green, Yellow, Orange, Red, Crimson and Far Red channels. The default crosstalk compensation accommodates a range of commonly used fluorophores. However, the QIAcuity Suite 3.0 goes one step further and lets users create custom crosstalk matrices for the first time (Figure 1A). With the ability to tune crosstalk compensation to the specific fluorophores in a given assay setup, the QIAcuity Software 3.0 provides users more freedom and added confidence to perform high multiplexed PCR on the QIAcuity System. In combination with the newly developed custom crosstalk compensation option and the QIAcuity High Multiplex Probe PCR Kit, users can detect targets in up to eight channels: six standard optical channels and two channels that make use of hydrolysis probes with long Stokes-shift dyes (LSS) (Figure 1B).



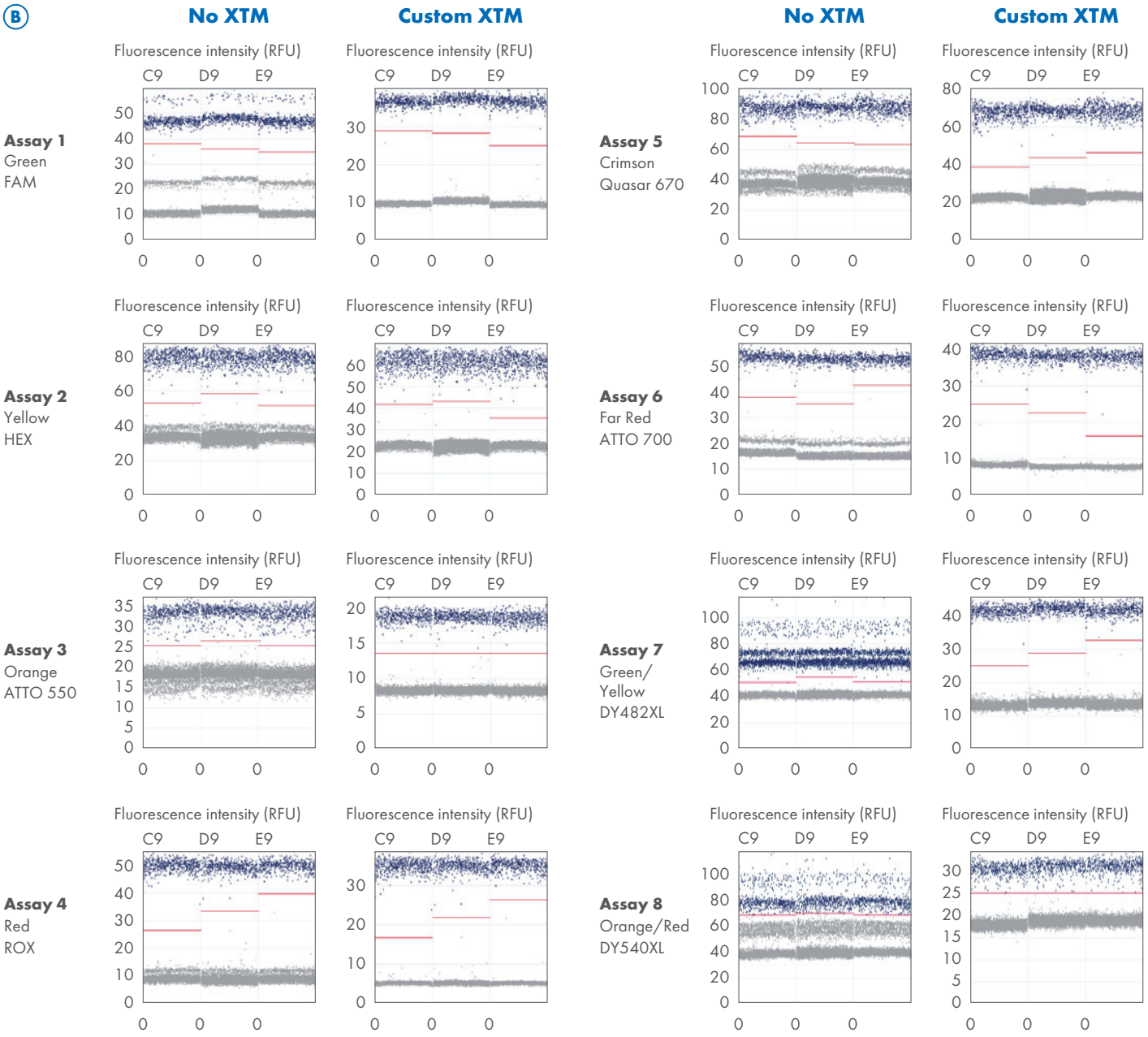
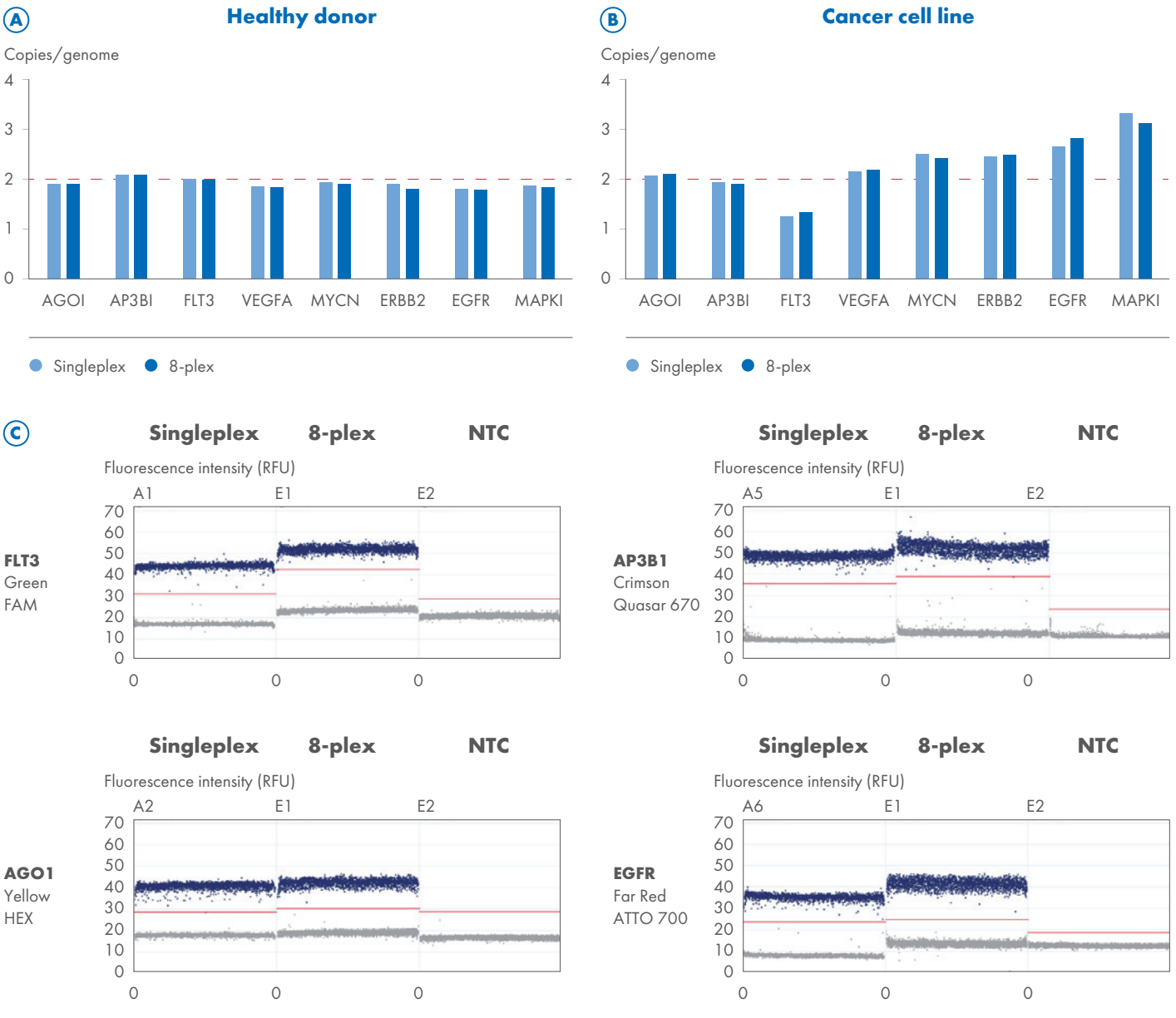


Figure 1. Enhanced crosstalk compensation in QIAcuity Software 3.0. In multiplexed dPCR reactions, crosstalk between neighboring QIAcuity channels can lead to false signals in 1D scatterplots. As the number of targets in a reaction increases, so does the risk of interference between channels. To address this, QIAcuity Software Suite 3.0 features enhanced and dynamic crosstalk compensation explicitly designed for these challenges. In reactions detecting up to six targets (Green, Yellow, Orange, Red, Crimson and Far Red channels), the default software settings (Standard XTM) effectively mitigate crosstalk across all channels (**A**). However, by training the crosstalk compensation with the unique spectral properties of a particular assay setup (Custom XTM), spurious signal between channels can be removed even more effectively. This is particularly evident in the Orange, Red and Crimson channels. When detecting eight targets (utilizing the six standard channels plus two long Stokes-shift channels) (**B**), QIAcuity Software 3.0 does not apply default crosstalk compensation (No XTM). In these 8-plex reactions, a custom crosstalk matrix is essential. If no custom matrix is employed, multiple bands of false-positive signals appear in the 1D scatterplots for each channel. Applying a custom crosstalk matrix eliminates interfering signal, resulting in clean 1D scatterplots that show only a single band of true positive signal.

When the elements of the QIAcuity platform tailored for high multiplexing come together, users reap the benefits of combining multiple assays in a single reaction while eliminating the associated risks and drawbacks. For example, interrogation of copy number variation (CNV) benefits from the greater degree of multiplexing now available on QIAcuity. With any CNV experiment, using reference targets for normalization is crucial. In fact, it is recommended to include at least two reference targets per reaction for best results. However, employing multiple reference assays reduces the number of gene of interest (GOI) targets that can be assayed in a reaction. Therefore, users are tempted to rely on a single reference gene, at the expense of obtaining more reliable data. Users do not need to make this sacrifice with the dPCR CNV Probe Assays and the QIAcuity High Multiplex Probe PCR Kit. Six gene of interest targets and two reference gene targets can be quantified in a single reaction to confidently capture important biological insights, such as genome instability resulting from cancer (Figure 2).



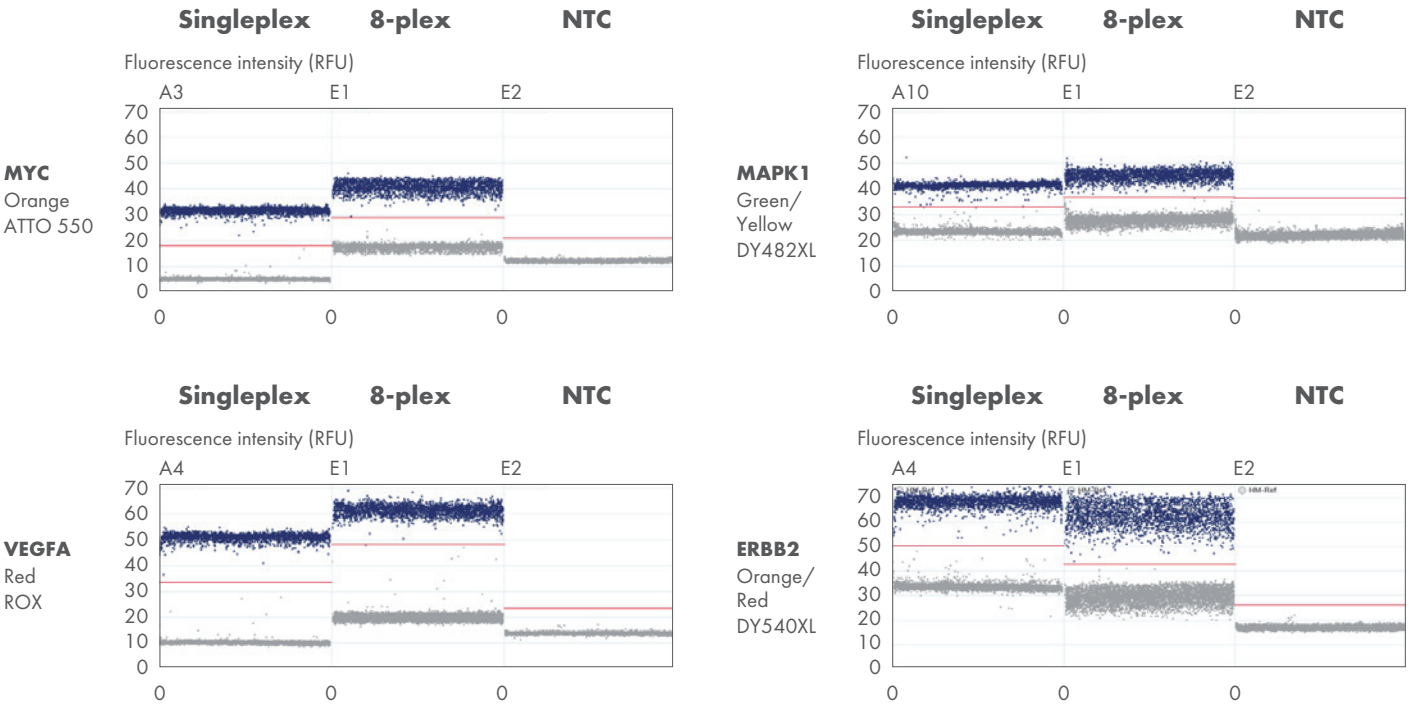
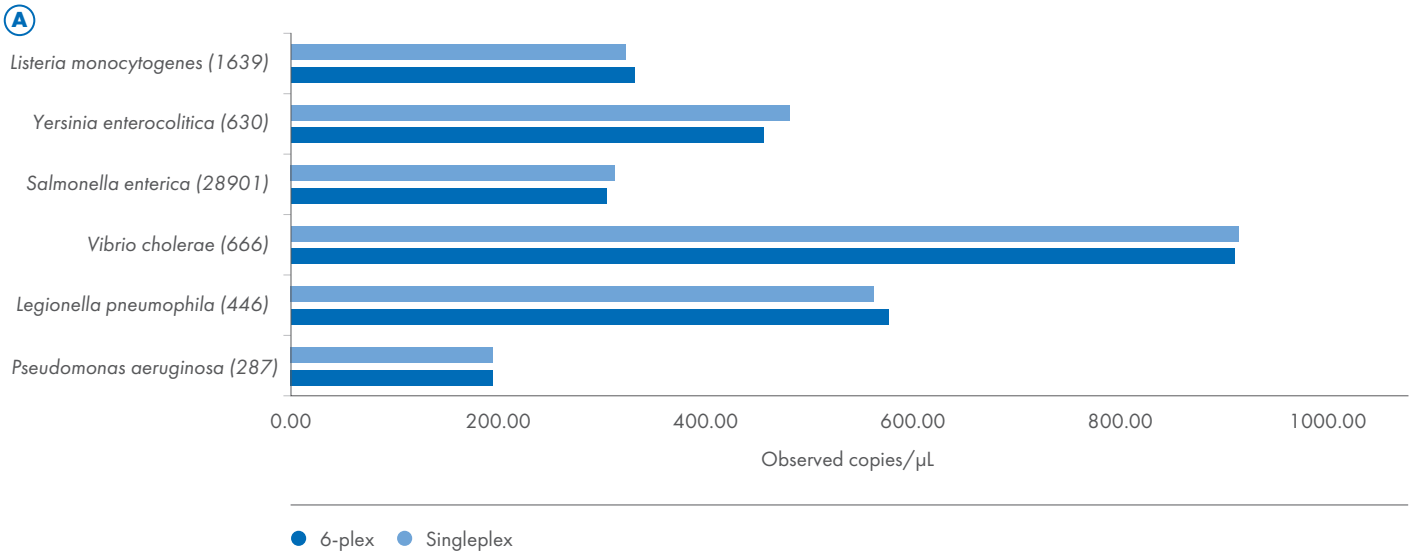


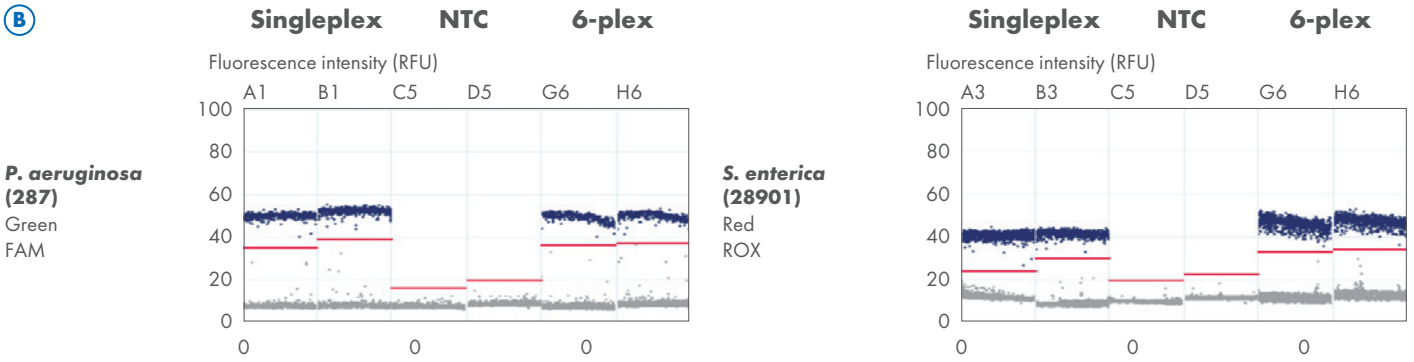
Figure 2. Detect more CNV targets with great confidence in a single reaction. Copy number variation (CNV) analysis benefits significantly from the 8-plex capabilities of the QIAcuity High Multiplex Probe PCR Kit combined with the QIAcuity Software Suite 3.0. In CNV studies, using multiple reference targets for normalization is crucial, with at least two references recommended per reaction for optimal results. Typically, this limits the number of gene of interest (GOI) targets that can be included in the same reaction. However, the new 8-plex capabilities of the QIAcuity system allow up to six GOI targets and two reference genes to be quantified in a single reaction, generating reliable data without compromise. To demonstrate this, 8-plex reactions were assembled to compare the copy number of six GOIs (FLT3, VEGFA, MYCN, ERBB2, EGFR, MAPK1) between a healthy human donor and the U-2 OS sarcoma cell line. The average copy number of the reference genes AGO1 and AP3B1 was used for normalization. All eight gene targets were also quantified in singleplex for comparison. In both singleplex and 8-plex reactions, the copy number of the six GOIs and the two reference genes in the healthy donor (**A**), was around 2, as expected (red dashed line). In contrast, the results from both the singleplex and 8-plex reactions revealed genome instability in the sarcoma cell line (**B**), with the GOI copy numbers varying from 1.25 to 3.3. Even the reference genes displayed slight instability. QIAcuity 1D scatterplots for the multiplex reactions with healthy donor genomic DNA show that amplification of targets between the singleplex and 8-plex reactions is equivalent (**C**).

Similarly, for users assaying microbial targets, the QIAcuity High Multiplex Probe PCR Kit can now help detect more targets in a single reaction than ever before. Additionally, the high-powered QIAcuity High Multiplex Probe PCR Kit has been designed to tolerate even higher levels of PCR inhibitors. This is to the benefit of wastewater surveillance programs, which are often tasked with screening inhibitory environmental samples for numerous pathogens.

The option to detect multiple targets in a single reaction provides wastewater surveillance programs with more comprehensive, economical and streamlined workflows.

To demonstrate the practicality of multiplexing pathogen targets with the QIAcuity High Multiplex Probe PCR Kit, six dPCR Microbial DNA Detection Assays pulled from the Wastewater 1 and Wastewater 2 bundles (*Pseudomonas aeruginosa* (287), *Legionella pneumophila* (446), *Vibrio cholerae* (666), *Salmonella enterica* (28901), *Yersinia enterocolitica* (630), *Listeria monocytogenes* (1639)) were run together in a single reaction (Figure 3). Combining the six targets required no prior optimization and produced quantifcation results that were consistent with singleplex reactions.





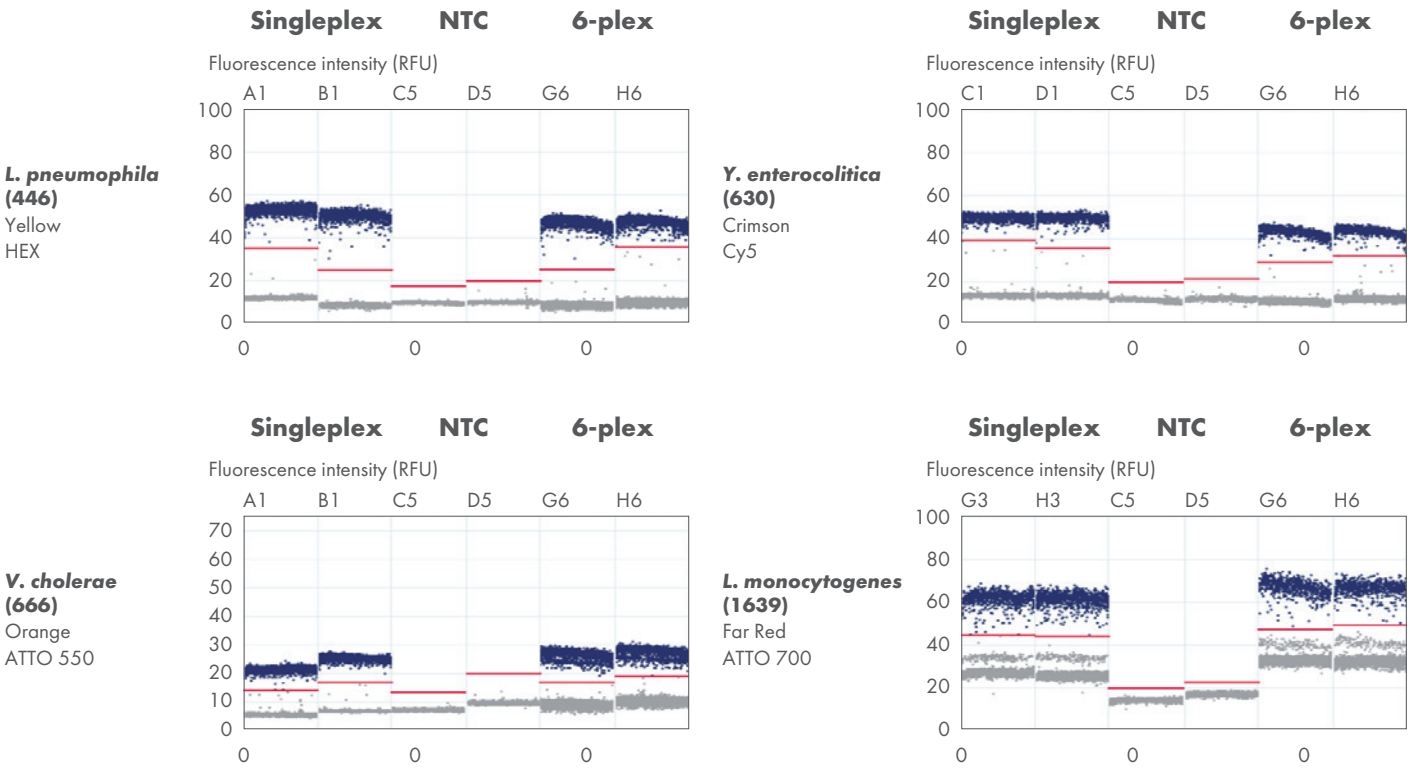


Figure 3. Streamline wastewater workflows by detecting more targets simultaneously. Six pathogen targets from the dPCR Microbial DNA Detection Assays Wastewater 1 and Wastewater 2 bundles, including *Pseudomonas aeruginosa* (287), *Legionella pneumophila* (446), *Vibrio cholerae* (666), *Salmonella enterica* (28901), *Yersinia enterocolitica* (630) and *Listeria monocytogenes* (1639), were successfully multiplexed in a single dPCR reaction without prior optimization. The results demonstrate consistent quantification between singleplex and multiplex reactions (**A**), showcasing the ability of the QIAcuity High Multiplex Probe PCR Kit to accurately detect multiple targets simultaneously. QIAcuity 1D scatterplots show equivalent amplification of targets between the singleplex and 6-plex reactions (**B**).

Summary

The significant advantages of highly multiplexed PCR regarding the efficient use of precious sample material, time savings and reagent reduction are desirable but come with risks. qPCR struggles with the technical challenges of high-level multiplexing, but the fundamental principles of digital PCR allow the QIAcuity platform to overcome these limitations. Recent innovations have pushed the possibilities of multiplexing on the QIAcuity even further. The QIAcuity Software Suite 3.0 software release allows for flexible crosstalk compensation, while the QIAcuity High Multiplex Probe PCR Kit lets users easily pursue even the most ambitious reaction setups in up to eight channels. The updated QIAcuity platform enhances workflows by offering higher sensitivity, greater target capacity and improved data reliability, making it a superior solution for high-order multiplexing.

For more information on QIAcuity digital PCR products, visit: **www.qiagen.com/applications/digital-pcr/products**

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.