Higher-order multiplexing on QIAcuity: 12-plex dPCR capabilities for detailed biological analysis

Introduction

Highly multiplexed PCR is a powerful technique that simultaneously detects numerous targets within a single reaction. By combining multiple assays in a single reaction, you can optimize sample usage, save time and minimize reagent consumption – accelerating the discovery of deeper biological insights. The benefits of multiplexing grow with the number of targets detected in a single reaction, making it highly advantageous to maximize the level of multiplexing.

Traditionally, the number of detection channels on a PCR instrument determines the number of detectable targets. In most scenarios, the physical constraints of hardware design and optics limit the amount of detection channels. However, digital PCR (dPCR) can employ a novel technique, termed amplitude-based multiplexing, to double the number of detectable targets in a single reaction – without requiring hardware modifications. Amplitude-based multiplexing leverages the fundamental principle of digital PCR, where reactions are partitioned into thousands of individual chambers, to detect two targets within a single channel. In amplitude-based multiplexing reactions, each partition can exist in one of four states: (1) negative for both targets, (2) positive for the first target only, (3) positive for the second target only or (4) positive for both targets.

Assay concentration largely determines the signal amplitude in dPCR 1D scatterplots. Therefore, when two assays for a given channel are added at different concentrations, the four possible partition states can be discriminated. In the 1D scatterplots, this manifests as four fluorescent signatures: three distinct clusters of positive signal with varying fluorescent intensity, corresponding to the three different states of positive partitions and one cluster of negative signal for the negative partitions

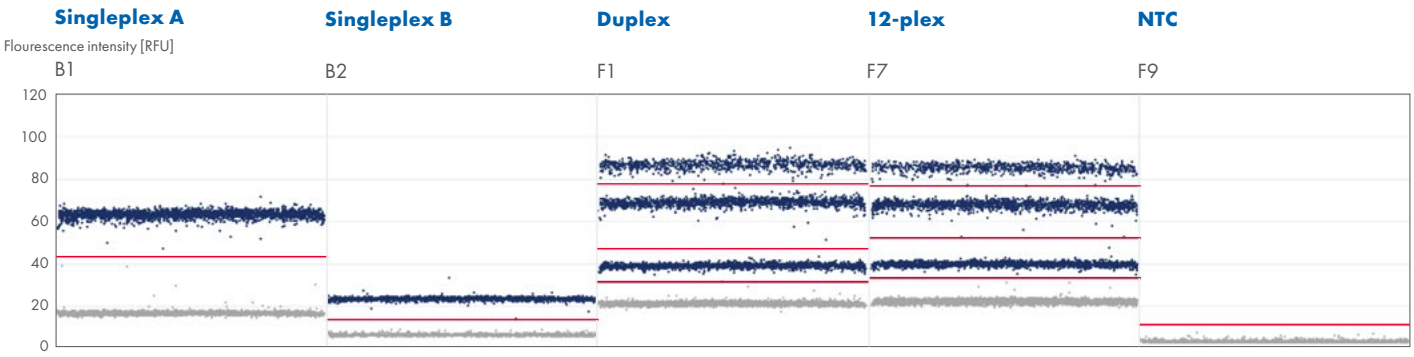

Figure 1. Amplitude-based multiplexing in QIAcuity Software Suite 3.1 enables precise target discrimination
1D scatterplots illustrating amplitude-based multiplexing in the Green channel in QIAcuity Software Suite 3.1 are shown. To detect two targets within the same channel, it is essential that the amplification signal of each target do not overlap with each other. This separation is achieved by using different concentrations of the respective assays in the reaction. In singleplex reactions in the Green channel, higher concentrations of Target A assay (well B1) produce a single band of positive signal (dark blue) that is brighter in comparison to amplification of Target B with lower assay concentrations (well B2). When the assays for targets A and B are combined in amplitude-based multiplex reactions detecting two (well F1) or twelve targets (well F7), three sharp clusters of positive signal are generated. Using QIAcuity Software 3.1, multiple thresholds (red lines) can be clearly set between these clusters. In wells F1 and F7, the lower signal clusters correspond to partitions positive for Target B only, while the middle signal clusters correspond to partitions positive for Target A only. The uppermost clusters correspond to partitions positive for both targets A and B. Negative partitions (no template) are presented as grey signal.

(Figure 1). This innovative approach effectively expands the capabilities of multiplexed dPCR, offering you a powerful tool for more efficient and comprehensive target detection.

To increase precision and reliability in amplitude-based multiplexing, the QIAcuity® dPCR platform seamlessly integrates advanced chemistry and intuitive software. The specialized chemistry comes in the form of the newly designed QIAcuity High Multiplex Probe PCR Kit. This kit features a custom-tailored PCR chemistry with a novel passive reference dye and custom-tailored PCR chemistry, specifically developed for amplitude-based multiplexing. We optimized the reaction buffer, increased the resistance to inhibitors and provide the highest DNA polymerase concentration among all QIAcuity mixes. This ensures exceptional performance, even for the most complex multiplexing setups. With the QIAcuity High Multiplex Probe PCR Kit, you can effortlessly perform higher-order multiplexing on the existing QIAcuity hardware.

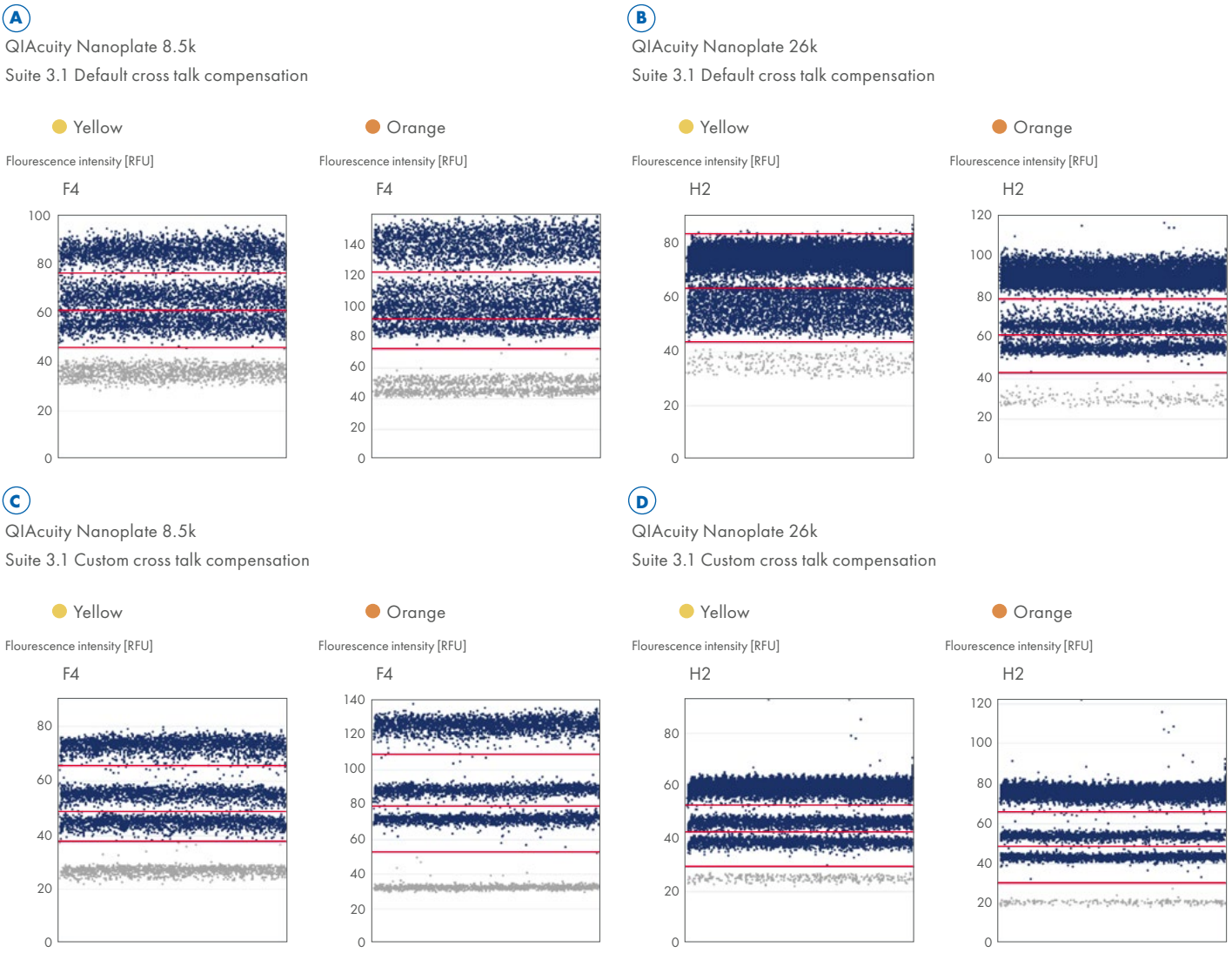

Figure 2. Custom cross talk matrices in QIAcuity Software Suite 3.1 greatly improve data quality in amplitude-based multiplexing reactions
Representative data for the Yellow and Orange channels are shown from an amplitude-based multiplexing reaction detecting twelve targets in the six standard QIAcuity channels. All twelve targets were present at a concentration of 3,000 copies/µl in the dPCR reaction. When the default cross talk compensation in QIAcuity Software Suite 3.1 is applied to the data, the three hallmark clusters of positive signal in the 1D scatterplots in QIAcuity Nanoplates 8.5k (**A**) and QIAcuity Nanoplates 26k (**B**) are sometimes barely distinguishable. In some instances multiple thresholds could not be set. However, when a custom cross talk matrix is applied to the data, the clusters of positive signal in both QIAcuity Nanoplates 8.5k (**C**) and QIAcuity Nanoplates 26k (**D**) are clearly separated. In all cases, multiple thresholds could be placed between the three clusters of positive signal.

The QIAcuity Software Suite 3.1 complements the High Multiplex Probe PCR Kit. This update simplifies the analysis of amplitude-based multiplexing experiments. In addition to the advanced cross talk features (introduced with version 3.0), you can now manually or automatically set multiple thresholds within a channel. Once thresholds are set, the software automatically calculates the concentrations of each target.

Amplitude-based multiplexing requires the clear separation of three signal clusters to set thresholds effectively. Cross talk between channels, however, may cause poorly distinguished signal populations. Applying a custom cross talk matrix to reactions employing amplitude-based multiplexing significantly enhances signal clarity. This ensures a well-defined separation of signal clusters across both the QIAcuity Nanoplates 8.5k and 26k, enhancing the reliability of your multiplexing results (Figure 2).

Using the QIAcuity Software Suite 3.1 and the QIAcuity High Multiplex Probe PCR Kit, you can detect up to twelve targets in the six standard optical channels across the entire dynamic range of the QIAcuity Nanoplates 8.5k and 26k. Theoretically, you can detect 170,000 copies of a given target per reaction on the QIAcuity Nanoplate 8.5k and up to 217,000 copies per reaction on the QIAcuity Nanoplate 26k. However, the upper limits of template input are impacted by the nanoplate format, choice of optical channel and the individual properties of each assay (for example, how tight each cluster of positive signal is in the 1D

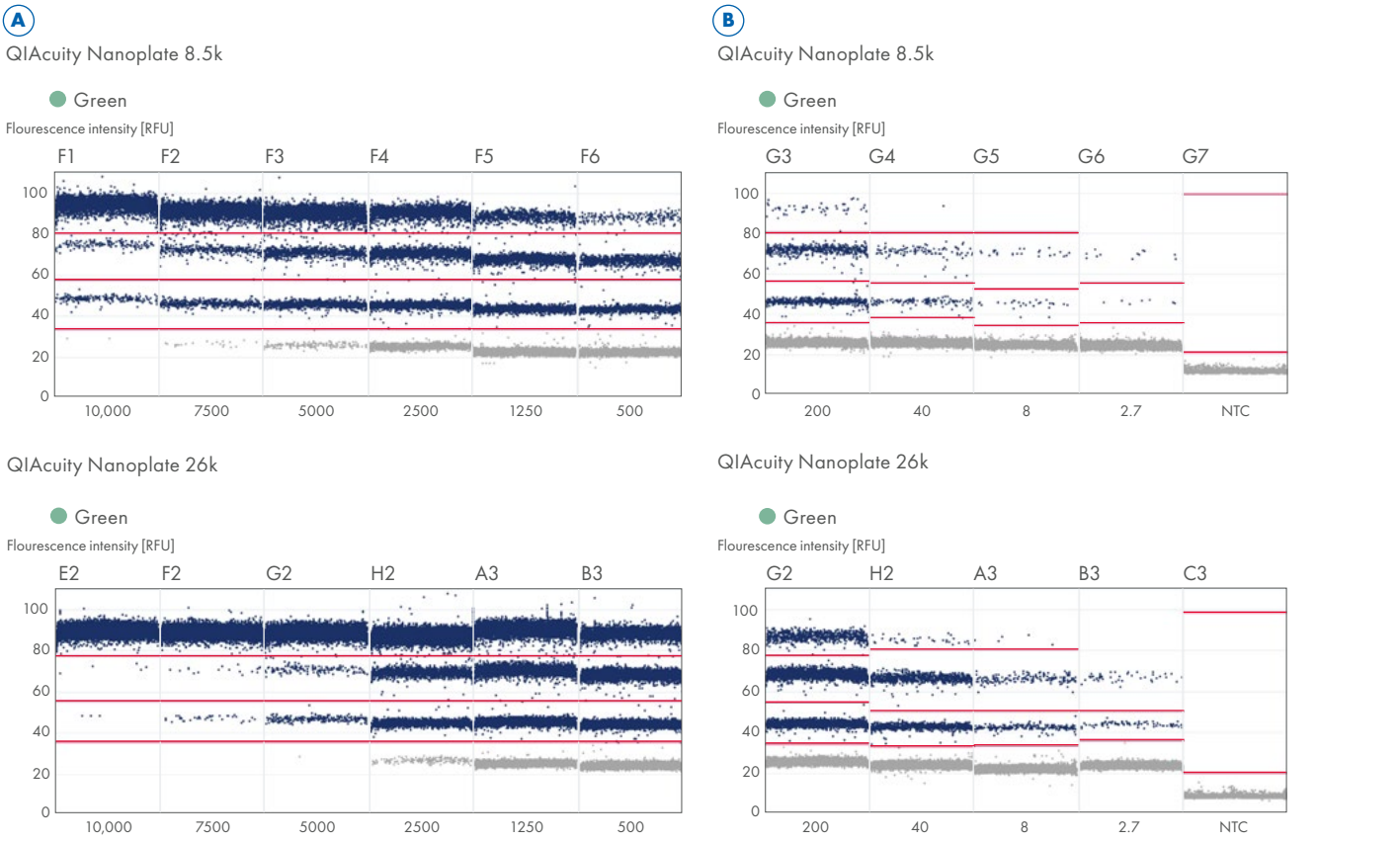

Figure 3. Intuitive data analysis from amplitude-based multiplexing: Depending on template concentration, one, two, or three clusters of positive signal will appear (**A**) If one or both targets in a given channel are present at high concentrations in amplitude-based multiplexing reactions (>7500 copies/µl in QIAcuity Nanoplates 8.5k, >5000 copies/µl in QIAcuity Nanoplates 26k), a significant portion of the positive signal will shift to "double positive" partitions with the highest fluorescent intensity. "Double positive" partitions are positive for targets of both assays. (**B**) In contrast, when one or both targets are present at low concentrations (<200 copies/µl in QIAcuity Nanoplates 8.5k, <40 copies/µl in QIAcuity Nanoplates 26k), few to no double-positive partitions are observed.

scatterplot). Ultimately, the ability to set thresholds between the discrete clusters of positive signal determines the upper limit for a given multiplex setup. If target concentrations are particularly high or low, the three clusters of positive signal that are the hallmark of amplitude-based multiplexing disappear. When one or both targets are present at high concentration (>7500 copies/µl in QIAcuity Nanoplates 8.5k, >5000 copies/µl in QIAcuity Nanoplates 26k), much or all of the signal will shift to positive clusters with the highest amplitude, corresponding to partitions positive for both targets. Conversely, when one or both targets are present at low concentrations, few to no double positive partitions will be present (<200 copies/µl in QIAcuity Nanoplates 8.5k, <40 copies/µl in QIAcuity Nanoplates 26k) (Figure 3).

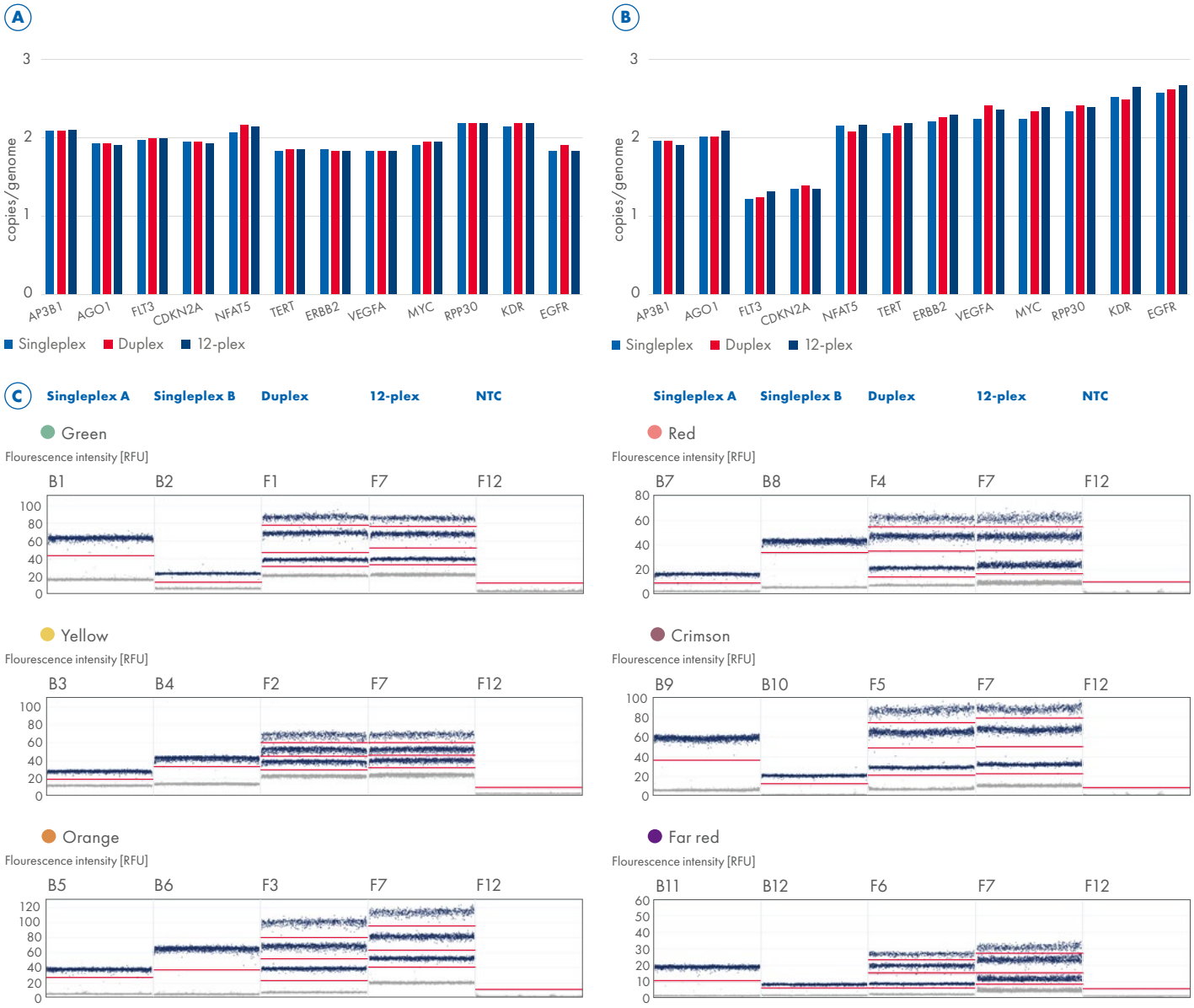

Figure 4. Detect more CNV targets with greater confidence in a single reaction.
Copy number variation (CNV) analysis benefits significantly from the 12-plex capabilities of the QIAcuity High Multiplex Probe PCR Kit combined with the QIAcuity Software Suite 3.1. To demonstrate this, 12-plex reactions were assembled to compare the copy number of ten GOIs (FLT3, CDKN2A, NFAT5, TERT, ERBB2, VEGFA, MYC, RPP30, KDR, and EGFR) 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 ten gene targets were also quantified in singleplex and duplex for comparison. In both singleplex, duplex and 12-plex reactions, the copy number of the ten GOIs and the two reference genes in the healthy donor was around 2, as expected (red dashed line) (**A**). In contrast, the results from both the singleplex, duplex and 12-plex reactions revealed genome instability in the sarcoma cell line, with the GOI copy numbers varying from 1.25 to 2.7 (**B**). QIAcuity 1D scatterplots for the singleplex, duplex, and 12-plex reactions with healthy donor genomic DNA demonstrate the equivalent performance of the assays between singleplex reactions and amplitude-based multiplex reactions (**C**). The 1D scatterplots also highlight the new option in QIAcuity Software Suite 3.1 to set multiple thresholds.

With the QIAcuity platform and products, you reap the benefits of high multiplexing while avoiding common risks. This is exemplified when interrogating copy number variation (CNV). For such experiments, guidelines recommend using at least two reference gene targets per reaction for normalization to get best results. However, to increase the number of gene of interest (GOI) targets per reaction, researchers are often tempted to rely on only one reference gene. This sacrifice is unnecessary with the QIAcuity platform. Using the dPCR CNV Probe Assays and the QIAcuity High Multiplex Probe PCR Kit (instead of Mix), you can confidently quantify 10 GOIs and two reference gene targets in a single reaction to capture important biological phenomena, such as genome instability resulting from cancer (Figure 4).

Figure 5. Streamline wastewater workflows by detecting more targets simultaneously.
(**A**) Twelve pathogen targets from the dPCR Microbial DNA Detection Assays Resistance Gene bundles (ACC-1, OXA-1055, CTX-M-15, sul1, vanC, QepA1, oprm, blaVIM-1, QnrB-1, OXA-58, CFE-1, OqxA) were successfully multiplexed in a single dPCR reaction. (**B**) QIAcuity 1D scatterplots for the singleplex, duplex, and 12-plex reactions demonstrate the equivalent performance of the assays between singleplex reactions and amplitude-based multiplex reactions. The 1D scatterplots also highlight the new option in QIAcuity Software Suite 3.1 to set multiple thresholds. The results demonstrate consistent quantification between singleplex and multiplex reactions, showcasing the ability of the QIAcuity High Multiplex Probe PCR Kit to accurately detect multiple targets simultaneously.

Similarly, if you are assaying microbial targets, you can now detect more targets than ever before in a single reaction with the QIAcuity High Multiplex Probe PCR Kit. This is particularly helpful if you are screening for numerous microbial signatures in parallel, as is the case with wastewater surveillance programs. 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, dPCR Microbial DNA Detection Assays targeting twelve antimicrobial resistance genes (ACC-1, OXA-1055, CTX-M-15, sul1, vanC, QepA1, oprm, blaVIM-1, QnrB-1, OXA-58, CFE-1, OqxA) were run together in a single reaction (Figure 5). Quantification of the twelve targets was consistent between singleplex, duplex, and 12-plex reactions, even when moderate rain was observed for some assays. It is important to note that when assaying microbial targets, especially those in environment samples, the naturally occurring diversity for a given DNA sequence can lead to heterogenous amplification efficiency. This manifests as rain or multiple clusters of positive signal in the 1D Scatterplots. Therefore, you should test your microbial assays and samples in singleplex reactions before proceeding with amplitude-based multiplexing experiments.

Summary

Highly multiplexed PCR provides substantial benefits, including enhanced sample efficiency, time savings and reduced reagent use. However, leveraging these advantages can be challenging due to the technical constraints of traditional methods like qPCR. Amplitude-based multiplexing with QIAcuity dPCR addresses these limitations by surpassing the typical multiplexing capacity of qPCR. By detecting up to 12 targets within a single reaction without any hardware changes, the QIAcuity platform realizes the full potential of highly multiplexed assays.

The synergy of advanced QIAcuity chemistry and software powers this breakthrough. The QIAcuity Software Suite 3.1 introduces flexible cross talk compensation and streamlined analysis for amplitude-based multiplexing, while the QIAcuity High Multiplex Probe PCR Kit simplifies even the most complex reaction setups. Together, these tools bring ease and precision to reactions looking to maximize target detection.

With improved sensitivity, increased target capacity and enhanced data reliability, the updated QIAcuity platform sets a new standard for high-order multiplexing, making it a powerful and reliable solution for advanced PCR workflows.

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