February 2025

QIAcuity® High Multiplex Probe PCR Kit Handbook

For quantification of up to 12 targets in a single reaction on the QIAcuity platform

Sample to Insight

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Kit Contents

QIAcuity High Multiplex Probe PCR Kit (1 mL) Catalog no.	250133
4x QIAcuity High Multiplex Probe PCR Mastermix (1 mL)	1 tube
QuantiNova Internal Control DNA dPCR	1 tube
RNase-Free Water	2 tubes

QIAcuity High Multiplex Probe PCR Kit (5 mL) Catalog no.	250134
4x QIAcuity High Multiplex Probe PCR Mastermix (1 mL)	5 tubes
QuantiNova Internal Control DNA dPCR	1 tube
RNase-Free Water	8 tubes

dPCR Instruments and Nanoplate Formats Compatible with the QIAcuity High Multiplex Probe PCR Kit

Table 1. dPCR Instruments and Nanoplate Formats Compatible with the QIAcuity High Multiplex Probe PCR Kit

Instruments	Instrument cat. nos.	Nanoplate formats	Nanoplate cat. nos.
QIAcuity One, 2plex	911001	8.5k 24-well	250011
		8.5k 96-well	250021
		26k 8-well	250031
		26k 24-well	250001
QIAcuity One, 5plex	911021	8.5k 24-well	250011
		8.5k 96-well	250021
		26k 8-well	250031
		26k 24-well	250001
QIAcuity Four, 5plex	911042	8.5k 24-well	250011
		8.5k 96-well	250021
		26k 8-well	250031
		26k 24-well	250001
QIAcuity Eight, 5plex	911052	8.5k 24-well	250011
		8.5k 96-well	250021
		26k 8-well	250031
		26k 24-well	250001

Shipping and Storage

The QIAcuity High Multiplex Probe PCR Kit is shipped on dry ice. Upon receipt, the kit should be stored protected from light at –30°C to –15°C in a constant-temperature freezer. Under these conditions, the kit is stable without showing any reduction in performance and quality, until the date indicated on the label. Before opening any tube, briefly centrifuge the tube to collect all material at the bottom.

Intended Use

The QIAcuity High Multiplex Probe PCR Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAcuity High Multiplex Probe PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Highly multiplexed PCR with the QIAcuity High Multiplex Probe PCR Kit

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, reduce 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.

dPCR is an ideal option for higher-order multiplexing

The QIAcuity digital PCR (dPCR) platform addresses and overcomes the challenges of high multiplexed 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 partitions positive for PCR amplification, QIAcuity dPCR delivers absolute quantification of 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 highly multiplexed qPCR.

Principle and procedure

QIAcuity High Multiplex Probe PCR Mastermix

The QIAcuity High Multiplex Probe PCR Mastermix uses a novel passive reference dye and specifically tailored PCR chemistry to let users conveniently perform higher-order multiplexing on existing QIAcuity hardware. The PCR buffer has been optimized for multiplexing while the

DNA polymerase is supplied at the highest concentrations in any QIAcuity mix. This tailored chemistry enables accurate quantification of up to 12 targets having widely differing abundance within a single well of the QIAcuity Nanoplate. This saves time, money, and reduces the amount of sample material needed.

Highly specific multiplexing with the QIAcuity High Multiplex Probe PCR Mastermix is made possible in part by a novel, antibody-mediated, hot-start mechanism. At low temperatures, the QuantiNova® DNA Polymerase is kept in an inactive state by the QuantiNova Antibody and a novel additive, QuantiNova Guard, which stabilizes the complex. This improves the stringency of the hot-start and prevents extension of nonspecifically annealed primers and primer dimers. Within 2 minutes of raising the temperature to 95°C, the QuantiNova Antibody and QuantiNova Guard are denatured, and the QuantiNova DNA Polymerase is activated, enabling the PCR amplification. The outstanding stability of the polymerase, even after extended storage at room temperature without the use of any cooling agent, makes the QIAcuity High Multiplex Probe PCR Mastermix ideal for high-throughput reaction setup.

QIAcuity High Multiplex Probe PCR Kit Requires QIAcuity Suite Software Version 3

The QIAcuity High Multiplex Probe PCR Kit works exclusively with the newly released QIAcuity Suite 3 or higher. Only version 3 of the QIAcuity Software Suite is able to detect the novel passive reference dye found in the QIAcuity High Multiplex Probe PCR Mastermix.

Additionally, QIAcuity Software Suite 3 offers refined cross talk features that are essential for getting the best results from highly multiplexed reactions. A refined default cross talk compensation is automatically applied to reactions targeting up to 6 targets in the Green, Yellow, Orange, Red, Crimson, and Far Red channels. The default cross talk compensation accommodates a range of commonly used fluorophores. However, the QIAcuity Software Suite 3 goes one step further and lets users create custom cross talk matrices for the first time. When using long Stokes-shift (LSS) dyes, applying a custom cross talk matrix is mandatory, as each LSS dye brings with it unique spectral properties that a default compensation matrix

cannot anticipate. Using custom cross talk compensation is highly recommended when performing amplitude-based multiplexing, as it results in the best separation between the positive clusters.

Two approaches to higher-order multiplexing with the QIAcuity High Multiplex Probe PCR Kit

Higher-order multiplexing can be performed with the QIAcuity High Multiplex Probe PCR Kit using 2 approaches. Users can detect one target per channel in up to 8 channels or up to 12 targets in 6 channels using amplitude-based multiplexing.

Detecting up to 8 targets per reaction (one target per channel)

Using the QIAcuity Software Suite 3.0 and any of the QIAcuity 5-plex instruments, users can detect one target per channel in up to 8 channels in a single reaction with the QIAcuity High Multiplex Probe PCR Kit.

If detecting no more than 6 targets (one target per channel), users are strongly encouraged to use the 6 standard optical channels available to the QIAcuity High Multiplex PCR Kit: Green, Yellow, Orange, Red, Crimson, and Far Red.

To arrive at 8 channels, users must combine the 6 standard optical channels available to the QIAcuity High Multiplex PCR Kit (Green, Yellow, Orange, Red, Crimson, and Far Red) with 2 of the 5 hybrid channels available for use with long Stokes-shift dyes (Green/Yellow, Yellow/Orange, Orange/Red, Red/Crimson, and Crimson/Far Red). Long Stokes-shift dyes contribute cross talk to multiple channels and are conversely impacted by the cross talk from multiple channels. Therefore, no single cross talk matrix can work with LSS dyes. By default, the QIAcuity Software Suite applies no cross talk compensation when hybrid channels are activated. Therefore, a custom cross talk matrix must be generated and applied to reactions using hybrid channels during analysis.

Assay concentration guidelines for detecting one target per channel in up to 8 channels

If detecting no more than 6 targets (one target per channel) in the 6 standard optical channels available to the QIAcuity High Multiplex PCR Kit (Green, Yellow, Orange, Red, Crimson, and Far Red), users should use the assay concentrations outlined in Table 2.

Table 2. Assay concentration guidelines for detecting one target per channel in the 6 standard QIAcuity channels with the QIAcuity High Multiplex Probe PCR Kit.

	User Supplied Assays	QIAcuity dPCR CNV Probe Assay	QIAcuity dPCR DNA Microbial Detection Assay	QIAcuity dPCR LNA Mutation Assay
Green	0.8 µM primer 0.4 µM probe	1x	1x	1x
Yellow	0.8 µM primer 0.4 µM probe	1x	1x	1x
Orange	0.8 µM primer 0.4 µM probe	1x	1x	1x
Red	0.8 µM primer 0.4 µM probe	1x	1x	1x
Crimson	0.8 µM primer 0.4 µM probe	1x	1x	1x
Far Red	0.8 µM primer 0.4 µM probe	1x	1x	1x

When detecting up to 6 targets in a single reaction, using the standard QIAcuity channels (Green, Yellow, Orange, Red, and Far Red) is highly recommended. When done in this manner, each of the 6 assays should be added to the reaction at the same concentration.

Table 3. Assay concentration guidelines for detecting one target per channel with hybrid channels and the 6 standard QIAcuity channels with the QIAcuity High Multiplex Probe PCR Kit

	User Supplied Assays	QIAcuity dPCR CNV Probe Assay	QIAcuity dPCR DNA Microbial Detection Assay	QIAcuity dPCR LNA Mutation Assay
Green	0.4 µM primer 0.2 µM probe	0.5x	1x	0.5x
Yellow	0.4 µM primer 0.2 µM probe	0.5x	1x	0.5x
Orange	0.2 µM primer 0.1 µM probe	0.25x	0.5x	0.25x
Red	0.4 µM primer 0.2 µM probe	0.5x	1x	0.25x
Crimson	0.4 µM primer 0.2 µM probe	0.5x	1x	0.5x
Far Red	0.4 µM primer 0.2 µM probe	0.5x	1x	0.5x
Green/Yellow	0.4 µM primer 0.2 µM probe	0.4 µM primer 0.2 µM probe	0.4 µM primer 0.2 µM probe	0.4 µM primer 0.2 µM probe
Orange/Red	1.6 µM primer 0.8 µM probe	1.6 µM primer 0.8 µM probe	1.6 µM primer 0.8 µM probe	1.6 µM primer 0.8 µM probe

When detecting targets in multiplex reactions that employ hybrid channels, different assay concentrations are recommended. This is to mitigate cross talk issues, as Long Stokes-shift dyes contribute cross talk to multiple channels and are themselves impacted by the cross talk from multiple channels. Currently, only the Green/Yellow LSS dye DY 482XL and Orange/Red LSS dye DY 540XL have been shown to work well with the QIAcuity system.

If detecting targets in the hybrid channels in combination with the 6 standard channels, different assay concentrations are recommended (see Table 3). This is due to the fact that long Stokes-shift dyes contribute cross talk to multiple channels and are conversely impacted by the cross talk from multiple channels. When assays in hybrid channels are yielding poor results (e.g., multiple bands in 1D scatterplots or quantification issues) please review the setup parameters of the corresponding "standard" channels. For example, if there is an issue with

an assay in the Orange/Red channel, adjusting the conditions for the assays in the Orange or Red channel may be required. Currently, only LSS dyes in the Green/Yellow and Orange/Red hybrid channels have been tested with the QIAcuity system.

Recommendations for long Stokes-shift (LSS) dyes

Assays labeled with long Stokes-shift dyes are not available from QIAGEN GeneGlobe. User-supplied assays labeled with LSS dyes can be ordered from Biomers GmbH (**www.biomers.net**).

Currently, only the following 2 LSS dyes have been shown to work well with the QIAcuity system:

- DY482XL for the Green/Yellow hybrid channel
- DY540XL for the Orange/Red hybrid channel

The following LSS dyes are NOT recommended for use with the QIAcuity:

- DY 520XL
- DY 521XL
- DY 526XL

Detecting up to 12 targets per reaction with amplitude-based multiplexing

Using the QIAcuity Software Suite 3.1 and any of the QIAcuity 5-plex instruments, users can detect 2 targets per channel in up to 6 of the standard optical channels available to the QIAcuity High Multiplex PCR Kit (Green, Yellow, Orange, Red, Crimson, and Far Red) in a single reaction.

With the QIAcuity One 2-plex instrument and QIAcuity Software Suite 3.1, users can detect up to 4 targets per reaction: 2 targets in the Green channel and 2 targets in the Yellow channel.

The detection of 2 targets per channel in dPCR relies on an approach termed amplitude-based multiplexing. This method leverages the fundamental principle of digital PCR, where reactions are partitioned into thousands of individual chambers, to detect 2 targets within a single channel, effectively doubling detection capacity. In amplitude-based multiplexing reactions, each partition can exist in one of 4 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. As assay concentration largely determines the signal amplitude in dPCR 1D Scatterplots, using different concentrations for 2 assays in a given channel can be used to discriminate between the 4 possible partition states. In the 1D Scatterplots, this manifests as 4 fluorescent signatures: 3 distinct clusters of positive signal with varying fluorescent intensity, corresponding to the 3 different states of positive partitions, and one cluster of negative signal for the negative partitions (Figure 1). In the QIAcuity Software Suite 3.1, users can set multiple thresholds between the clusters of positive signal, which are then used by the suite to automatically calculate the concentrations of both targets in each channel.

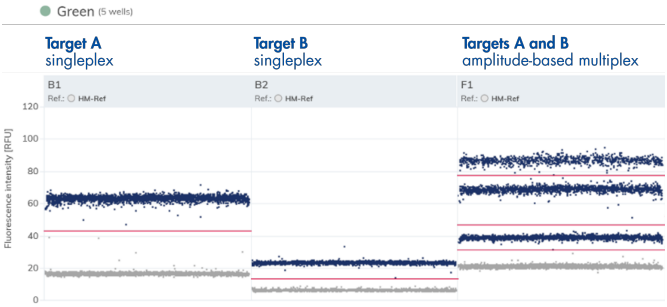


Figure 1. Example of 1D Scatterplots with amplitude-based multiplexing in QIAcuity Software Suite 3.1. Assays detecting 2 different targets (Targets A and B) were run in the Green channel. When run in singleplex reactions, assays for Target A (well B1) and Target B (well B2) each generate a single, sharp band of positive signal (dark blue). Because Target A was amplified with a higher concentration of assay compared to Target B, the amplitude of signal for Target A is greater than that for Target B. When the assays for targets A and B are combined in an amplitude-based multiplex reaction (well F1), 3 sharp clusters of clearly separated positive signal are generated. Using QIAcuity Software 3.1, multiple thresholds (red lines) can be set between these clusters. In the example shown in well F1, the lower cluster of signal corresponds to partitions positive for Target B only, while the middle cluster of signal corresponds to partitions positive for Target A only. The uppermost cluster corresponds to partitions positive for both targets A and B. Partitions negative for template are presented as gray signal.

Assay concentration and imaging setting guidelines for amplitude-based multiplexing

Differentiating the amplification of 2 targets within the same channel relies on the varying amplitude of fluorescent signal generated by each assay during the PCR reaction. Fluorescent amplitude in PCR is largely determined by the concentration of each assay in the reaction. Therefore, the concentration of assays must be carefully considered and optimized so that each target generates a signal of distinct amplitude that does not overlap with the other target. The assay concentrations outlined in Table 4 can be used as a starting point when performing amplitude-based multiplexing for the first time. As every assay is unique, users may need to adjust assay concentrations depending on their setup. Refer to Appendix B on page 56 for further details.

Amplitude-based multiplexing also benefits from positive signal that is not too bright, as this lowers the impact of optical cross talk on the analysis. In particular, the interplay between the Yellow, Orange, and Red channels can significantly impact the quality of signal separation in those channels. In our experience, lower signal intensities in the Yellow, Orange, and Red channels can help mitigate these challenges. As a guideline for amplitude-based multiplexing reactions, we have provided a table with the recommended maximum fluorescence level for each QIAcuity channel before custom cross talk compensation (Table 5). Achieving these RFU values will be determined by the particular assays used and imaging settings. Therefore, deviating from the default QIAcuity imaging settings may be required.

Table 4. Assay concentration guideline for detecting up to 12 targets in 6 channels using amplitude-based multiplexing with the QIAcuity High Multiplex Probe PCR Kit

	User Supplied Assays		QIAcuity dPCR CNV Probe Assay		QIAcuity dPCR MicrobialDNA Detection Assays*	
	Assay 1	**Assay 2**	**Assay 1**	**Assay 2**	**Assay 1**	**Assay 2**
Green			**0.5x**	**0.25x**	**1 x**	**0.5x**
	0.4 µM primer	0.2 µM primer	0.4 µM primer	0.2 µM primer	0.6 µM primer	0.3 µM primer
	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe
Yellow			**0.5x**	**0.25x**	**1 x**	**0.5x**
	0.4 µM primer	0.2 µM primer	0.4 µM primer	0.2 µM primer	0.6 µM primer	0.3 µM primer
	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe
Orange			**0.5x**	**0.25x**	**1 x**	**0.5x**
	0.4 µM primer	0.2 µM primer	0.4 µM primer	0.2 µM primer	0.6 µM primer	0.3 µM primer
	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe
Red			**0.5x**	**0.25x**	**1 x**	**0.5x**
	0.4 µM primer	0.2 µM primer	0.4 µM primer	0.2 µM primer	0.6 µM primer	0.3 µM primer
	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe
Crimson			**0.5x**	**0.25x**	**1 x**	**0.5x**
	0.4 µM primer	0.2 µM primer	0.4 µM primer	0.2 µM primer	0.6 µM primer	0.3 µM primer
	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe	0.2 µM probe	0.1 µM probe
Far Red			**1x**	**0.5x**	**2x**	**1x**
	0.8 µM primer	0.4 µM primer	0.8 µM primer	0.4 µM primer	1.2 µM primer	0.6 µM primer
	0.4 µM probe	0.2 µM probe	0.4 µM probe	0.2 µM probe	0.4 µM probe	0.2 µM probe

To detect 2 targets per channel with amplitude-based multiplexing, multiple clusters of non-overlapping positive signal must be generated within each channel. This is achieved by varying the concentration of the assays used to detect the targets. The use of amplitude-based multiplexing with hybrid channels and LSS dyes is not recommended. As mutation detection assays typically do not generate sharp bands of positive signal, amplitude-based multiplexing for mutation detection is not recommended.

* When using QIAcuity dPCR Microbial DNA Detection Assays for amplitude-based multiplexing reactions, the lyophilized assays can be resuspended in smaller volumes to generate more concentrated stocks (e.g., 40x). If 20x assay stocks are used, there will be less room to add sample in the reactions.

Table 5. Guidelines for maximum RFU level to achieve best results with amplitude-based multiplexing reactions

	Recommended maximum relative fluorescence unit (RFU) levels before custom cross talk compensation
Green	100 RFU
Yellow	80 RFU
Orange	80 RFU
Red	80 RFU
Crimson	100 RFU
Far Red	100 RFU

The clarity of 1D Scatterplots in amplitude-based multiplexing reactions is best when cross talk between channels in minimized. This can be achieved in part by managing the brightness of positive signal prior to custom cross talk compensation. The optical cross talk between the Yellow, Orange, and Red channels in particular can greatly influence the quality of signal separation in amplitude-based multiplexing reactions. By keeping signal intensities in the Yellow, Orange, and Red channels lower than in other channels, cross talk issues can be minimized. The RFU values listed above serve as guidelines for the maximum brightness of positive signal for each QIAcuity channel before applying custom cross talk compensation. As each reaction setup is unique, users may need to adjust these settings according to their needs.

Custom cross talk compensation with QIAcuity Software Suite 3

The amount of cross talk users observe in a QIAcuity reaction will depend on multiple factors. For example, the concentration of the fluorescently labeled hydrolysis probes, the type of fluorescent dyes attached to the hydrolysis probes, and the chosen imaging settings all have an influence on cross talk between channels. When the recommended dyes and imaging settings are used in a QIAcuity dPCR reaction, the QIAcuity Software Suite is equipped to automatically compensate for unwanted cross talk between neighboring channels (Green, Yellow, Orange, Red, Crimson, and Far red).

However, there are circumstances when a custom cross talk compensation should be used. For example, if using dyes that are not recommended by QIAGEN in any of the Green, Yellow, Orange, Red, Crimson, or Far red channels, the default QIAcuity cross talk compensation will not suffice. The same also applies for reactions that use long Stokes-shift (LSS) dyes. In these special instances, the new custom cross talk feature of the QIAcuity Software Suite version 3 must be used for optimum performance. When performing amplitude-based multiplexing, custom cross talk compensation is not required but it is highly recommended, as it ensures the clearest separation between the multiple bands of signal produced in each channel.

How a custom cross talk matrix (CXTM) works

To correct for optical cross talk in a multiplex reaction, the fluorescent properties of each fluorophore must be considered individually. Therefore, for a given multiplex reaction, each of the assays must be run in singleplex as well. In the case of amplitude-based multiplex reactions, a single reaction containing both of the assays for a given channel should be run (e.g., both Green assays should be run together in a single reaction). This lets the image analysis algorithm capture the fluorescence profile of each dye separately. The passive reference dye also contributes to cross talk and must also be taken into consideration. Therefore, wells containing master mix without any fluorescently labeled assays must also be added to the plate.

The custom cross talk feature employs user defined reaction mixes for its calculations. When detecting one target per channel, users must add reaction mixes for all singleplex reactions to a plate in order to define a custom cross talk matrix. When detecting 2 targets per channel, users must add reaction mixes for all amplitude-based duplex reactions to a plate in order to define a custom cross talk matrix. A reaction mix containing no assays must also be added. Mixes can be defined using the Plate configurator and then added to the plate via the Plate layout environment. After these steps have been successfully completed, the custom cross talk matrix configurator can be accessed via "Create CXTM" in the Analysis menu or in the Plate Overview.

While creating custom cross talk matrices, users are prompted to inspect, and adjust if necessary, the threshold settings for the singleplex and/or duplex reactions that will inform the cross talk compensation. Thresholds must be set such that positive signal is distinguished from the negative signal. For the amplitude-based duplex reactions, this step is especially critical, as the thresholds must be set below all 3 clusters of positive signal (Figure 2). If the threshold is not set in this way, cross talk compensation will be incorrect.

Custom cross talk matrices can be shared between plates. However, if importing a custom cross talk matrix to a new run from an existing template, all of the conditions between the two must be the same: assays used, assay concentrations, and imaging settings. If any one of the parameters between the two is different, custom cross talk compensation will not be correct.

Refer to the technical note "Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity Software Suite 3.0 and 3.1" for more details on how to create a custom cross talk matrix.

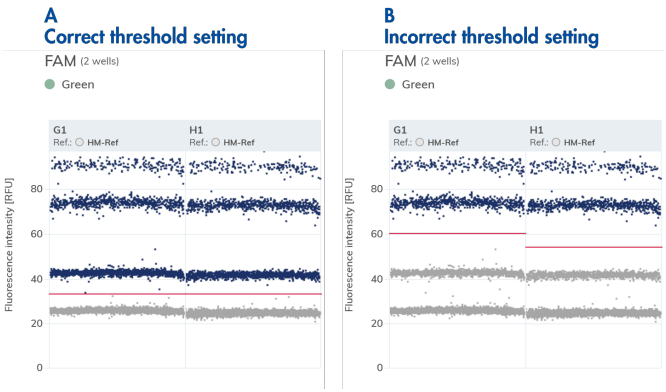


Figure 2. Correct threshold settings are essential to generate accurate custom cross talk matrices for amplitude-based multiplexing reactions. While generating custom cross talk matrices, users should review and modify threshold settings, particularly for amplitude-based multiplexing reactions. Thresholds are required to separate positive signal from negative signal, which informs the cross talk compensation for each channel. In amplitude-based duplex reactions, thresholds must be positioned below all 3 clusters of positive signal **(A)** to achieve correct cross talk compensation. Failure to set thresholds appropriately **(B)** can lead to errors in cross talk compensation.

Amplitude-based multiplexing reactions benefit greatly from custom cross talk matrices

The application of custom cross talk matrices is highly recommended for amplitude-based multiplex reactions. When 1D scatterplots for amplitude-based multiplex reactions with and without custom cross talk compensation in QIAcuity Software Suite 3.1 are compared, the differences in data quality are noticeable (Figure 3). Custom cross talk compensation ensures the best separation between the multiple clusters of positive signal for each channel, particularly in 26k Nanoplates. This in turn allows users to more easily set thresholds.

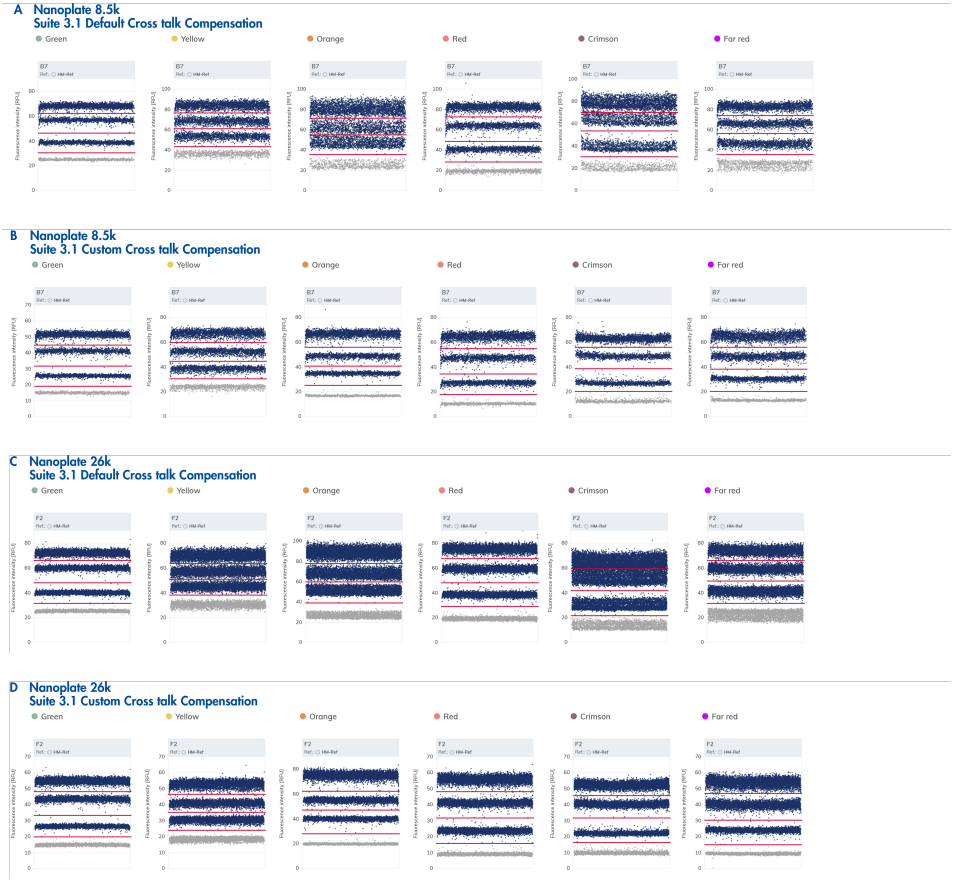


Figure 3. Custom cross talk matrices in QIAcuity Software Suite 3.1 greatly improve data quality in amplitude-based multiplexing reactions. Representative data are shown from an amplitude-based multiplexing reaction detecting 12 targets in the 6 standard QIAcuity channels. All 12 targets were present at a concentration of 3000 copies/µL in the dPCR reaction. When the default cross talk compensation in QIAcuity Software Suite 3.1 is applied to the data, the 3 hallmark clusters of positive signal in the 1D Scatterplots in **(A)** 8.5k Nanoplates and **(C)** 26k Nanoplates are sometimes barely distinguishable in the Yellow, Orange, and Crimson channels, such that multiple thresholds could not be set. When a custom cross talk matrix is applied to the data, the positive signal in both **(B)** 8.5k and **(D)** 26k Nanoplates shows much clearer separation. In all channels, multiple thresholds could be set for the 3 clusters of positive signal.

It is important to note that when creating a custom cross talk matrix for reactions employing amplitude-based multiplexing, the assays used for a given channel must be labeled with the same fluorophore. Mixing hydrolysis probes with different fluorophores in the same channel (e.g., ROX and TexasRed in the Red channel) will result in suboptimal cross talk compensation.

Sample Input

Input guidelines for detecting up to 8 targets per reaction (one target per channel)

Total expected copies per target must lie within the dynamic detection range of the QIAcuity Digital PCR Nanoplate. Up to 170,000 copies of each target per reaction can be detected when using the 8.5K Nanoplate, while up to 217,000 copies of each target per reaction can be quantified with the QIAcuity 26K Nanoplate. This is based on the calculations shown in Table 6.

Table 6. Nanoplates and their maximal copy numbers with hydrolysis probe chemistries

Nanoplate	Upper limit of copies per partition	Analyzed volume (µL)	Total reaction volume (µL)	Max copy number per analyzed volume	Estimated max copy number per reaction
8.5k	5	Approx. 2.6	12	42,500	170,000
26k	5	Approx. 22.0	40	130,000	217,000

If the haploid genome size of the organism studied is known, the correlation between mass input of gDNA and the resulting copy number (for a single-copy gene) can be easily calculated using the following formula:

Size of the haploid genome (bp) x average mass of a base pair (1.096×10^{-21} g/bp)

For the human genome with size of ca. 3.1×10^9 bp, the calculation is as follows:

3.1×10^9 bp x 1.096×10^{-21} g/bp = 3.3×10^{-12} g = 3.4 pg per haploid genome

For human, mouse, and rat genomes, the copy number of single copy genes corresponding to different masses of genomic DNA of are presented in Table 7.

Table 7. Conversion of DNA mass to copy number for human, mouse, and rat genomes

Organism	Haploid Genome Size*	Approximate copy number of single-copy genes (1 copy per haploid genome) corresponding to the indicated amount of genomic DNA (gDNA)			
		100 ng	10 ng	1 ng	0.1 ng
Human (*Homo sapiens*)	3.1 x 10^9 bp	29,000	2900	290	29
Mouse (*Mus musculus*)	2.7 x 10^9 bp	34,000	3400	340	34
Rat (*Rattus norvegicus*)	2.6 x 10^9 bp	35,000	3500	350	35

* Genome sizes sourced from **www.ensembl.org**. Information is current as of November 2023. Genome size may change upon updates of the respective databases.

It is recommended to set up reactions with template input amounts resulting in 0.5–2 DNA copies/partition. This means:

- Approximately 17,000– 100,000 copies per 12 µL 8.5k Nanoplate reaction (approximately 56–330 ng of human gDNA for a single-copy gene)
- Approximately 22,000– 130,000 copies per 40 µL 26k Nanoplate reaction (approximately 73–430 ng of human gDNA for a single-copy gene).

However, the average number of copies per partition must not exceed 5.

If the copy number input cannot be determined before starting the experiment, it is recommended to perform an initial titration experiment using the unknown template in 2–4 tenfold dilutions to determine the optimal range for subsequent analyses.

Input guidelines for detecting up to 12 targets per reaction with amplitude-based multiplexing

When performing amplitude-based multiplexing, total expected copies per target must lie within the dynamic detection range of the QIAcuity Digital PCR Nanoplate. Theoretically, up to 170,000 copies of each target per reaction can be detected when using the 8.5K Nanoplate, while up to 217,000 copies of each target per reaction can be quantified with the QIAcuity 26K Nanoplate. However, the upper limits of template input for amplitude-based multiplexing are impacted by Nanoplate format, choice of optical channel, and the individual properties of each assay (e.g., how tight the cluster of positive signal is in the 1D scatterplot). Ultimately, the ability to set thresholds between the discrete clusters of positive signal within a channel will determine the upper limit for a given multiplex setup.

It is important to note that in scenarios where target concentrations are particularly high or low, the 3 clusters of positive signal that are the hallmark of amplitude-based multiplexing may not be present. When one or both targets are present at high concentrations (>7500 copies/µL in 8.5k Nanoplates, >2500 copies/µL in 26kNanoplates), most or all of the signal will shift to the highest-amplitude positive clusters, which represent partitions positive for both targets (i.e., double- positive partitions). In this scenario, few or no negative partitions will be present. However, this does not mean that the reaction will have reached saturation. For a given target in a amplitude-based duplex reaction, saturation occurs when there are no single-positive partitions for the second target as well as no negative partitions. Single-positive partitions for the second target, along with negative partitions, act as the negative partitions required by Poisson statistics to calculate the concentration of the first target.

When one or both targets are present at low concentrations, double positive partitions will not be present (<200 copies/µL in 8.5k Nanoplates, <50 copies/µL in 26k Nanoplates) (Figure 4). In both of these instances, users must be careful when setting the thresholds for their reactions. Running a positive control where both targets for a given channel are present at concentrations between 500 and 1000 copies/µL each is highly recommended. At these

concentrations, 3 clear clusters of positive signal will be generated in both 8.5k and 26k Nanoplate formats, which can serve as a guide for thresholding signal in other wells.

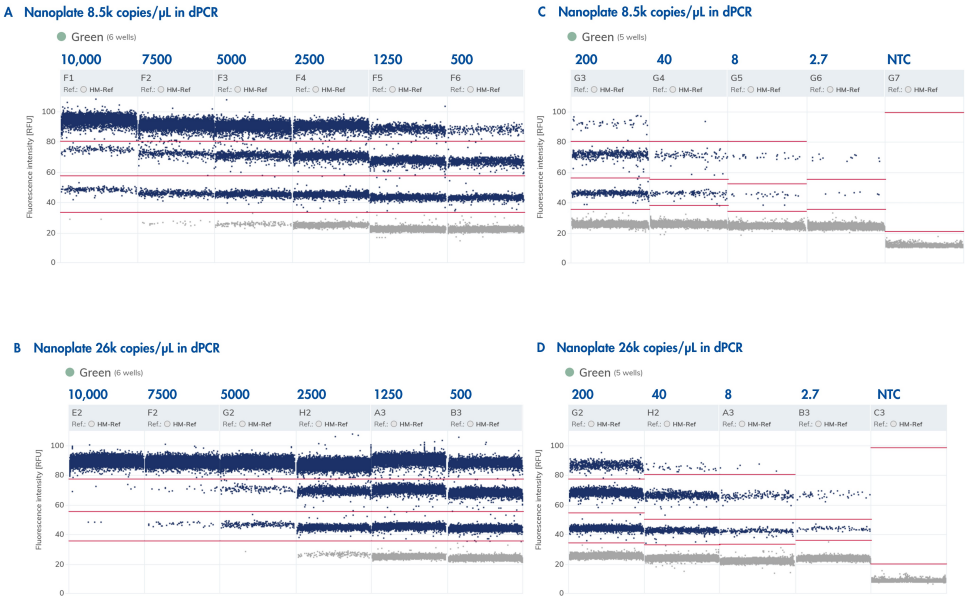


Figure 4. Depending on template concentration, one, 2, or 3 clusters of positive signal will appear. If one or both targets in a given channel are present at a high concentration in amplitude-based multiplexing reactions (**(A)** >7500 copies/µL in 8.5k Nanoplates, **(B)** >2500 copies/µL in 26k Nanoplates), a significant portion of positive signal will shift to "double positive" partitions with the highest fluorescent intensity. "Double positive" partitions are positive for targets of both assays. In contrast, when one or both targets are present at a low concentrations (**(C)** <200 copies/µL in 8.5k Nanoplates, **(D)** <50 copies/µL in 26k Nanoplates), few to no double-positive partitions will be observed.

Restriction enzyme digestion

DNA samples with ≥20 kb average length (e.g., gDNA purified via spin column with silica membrane, or salting out method) should be fragmented by restriction digestion before partitioning. Enzymatic fragmentation of larger DNA ensures even distribution of template

throughout the QIAcuity Nanoplate for accurate and precise quantification. Restriction digestion is not required for highly fragmented DNA (e.g., FFPE DNA or circulating DNA) or cDNA. Care should be taken to use enzymes that will not cut within the amplified sequence. Visit **geneglobe.qiagen.com** for information on QIAGEN's dPCR CNV Probe, dPCR Microbial DNA Detection, and dPCR LNA Mutation assays.

For convenience, restriction digestion can be performed directly in the QIAcuity reaction mix. The restriction enzymes listed in Table 8 have been validated to digest up to 700 ng of human genomic DNA in 10 min at room temperature in the QIAcuity High Multiplex Probe PCR Master Mix without impairing the subsequent PCR amplification. The restriction enzymes are simply added to the mix during the reaction setup. Alternatively, genomic DNA may be fragmented with restriction enzymes separately, before reaction setup. The fragmented template is then added to the mix during reaction setup.

Table 8. Validated restriction enzymes

6-cutter restriction enzymes		4-cutter restriction enzymes	
*Eco*RI	0.25 U/µL EcoRI-HF®, NEB® 0.025 U/µL Anza™ 11 EcoRI, Thermo Fisher Scientific (TFS)	*Alu*I	0.025 U/µL AluI, NEB 0.025 U/µL Anza 44 AluI, TFS
*Pvu*II	0.025 U/µL PvuII, NEB 0.025 U/µL Anza 52 PvuII, TFS	*CviQ*I	0.025 U/µL CviQI, NEB 0.025 U/µL Csp6I (CviQI), TFS
*Xba*I	0.025 U/µL Anza 12 XbaI, TFS	*Hae*III	0.025 U/µL BsuRI (HaeIII), TFS

Important: To ensure that your DNA samples are fully digested, the particular versions of the restriction enzymes from the indicated suppliers must be used.

QuantiNova Internal Control DNA dPCR

The QuantiNova (QN) Internal Control (IC) DNA dPCR is a synthetic DNA that has been prequantified with QIAcuity dPCR. The exact concentration is printed on the tube label. The QN IC DNA supplied with the kit can optionally be used to monitor for PCR amplification.

When used by itself, the QN IC DNA is intended to report instrument failures, chemistry failures, and errors in assay setup. When added to reactions containing sample DNA, quantification of QN IC DNA can be used to assay for the presence of dPCR inhibitors. To detect PCR inhibition, users must also assemble at least one dPCR reaction that contains only QN IC DNA for comparison purposes.

The QN IC DNA is detected with a 200 bp amplicon. The primer sequences used to amplify QN IC DNA have been bioinformatically validated for non-homology against hundreds of eukaryotic and prokaryotic organisms. Additionally, they have been experimentally tested against a multitude of human, mouse, and rat RNA samples from a variety of tissues and cell lines. To detect QN IC DNA in dPCR reactions with the QIAcuity High Multiplex Probe PCR Kit, purchase the QuantiNova IC Probe Assay (200) (cat. no. 205813), which can be detected in the Yellow channel on QIAcuity instruments. Do not purchase the QuantiNova IC Probe Assay Red 650 (500) (cat. no. 205824), as it is not optimized to work with QIAcuity.

Users are recommended to add the QN IC DNA to their QIAcuity reactions at a final concentration of 100–1000 copies/µL. The RNase-Free Water supplied with the kit can be used to prepare dilutions from the QN IC DNA stock.

Purification of DNA

The quantity and purity of a DNA sample can affect the performance of the QIAcuity dPCR. It is highly recommended to refer to the following guidelines prior to setting up dPCR reactions.

DNA purification for Copy Number Variation (CNV) Analysis

When starting with purification of genomic DNA, an appropriate purification method should be used depending on the starting material and desired amount of purified genomic DNA. The kits listed below are recommended for genomic DNA purification from various types of starting material for use with QIAGEN's dPCR Copy Number Variation (CNV) Probe Assays (Table 9). DNA purification can be carried out according to the kit instructions.

Table 9. Recommended DNA purification kits for use with QIAGEN's dPCR Copy Number Variation (CNV) Probe Assays

Starting material	Purification method	Cat. no.
Fresh or frozen tissues, cultured cells	QIAamp DNA Mini Kits	51304 and 51306
	DNeasy® Blood & Tissue Kits	69506, 69504, 69582, and 69581
	EZ1& EZ2™ DNA Investigator Kit	952034
	EZ1&2 DNA Tissue Kit	953034
	Blood & Cell Culture DNA Mini, Midi, or Maxi Kit	13323, 13343, or 13362, respectively
	FlexiGene® DNA Kit	51206
	PAXgene® Tissue DNA Kit	767134
FFPE tissues	QIAamp® DNA FFPE Tissue Kit	56404
	QIAamp DNA FFPE Advanced UNG Kit	56704
cfDNA from human plasma or serum	QIAamp DNA Blood Mini or Maxi Kits	51106, or 51194 and 51192, respectively
	PAXgene Blood DNA Kit	761133
	QIAamp ccfDNA/RNA Kit	55184
	EZ1&2 ccfDNA Kit	954854
	QIAamp MinElute ccfDNA Midi and Mini Kits	55284 and 55204, respectively
Other biological samples	Contact QIAGEN Technical Service	

Important: Do not use water treated with diethyl pyrocarbonate (DEPC).

Important: Presence of RNA in the sample preparations will result in inaccurate DNA concentration measurements. Removing RNA contamination with an RNase A digestion is highly recommended.

Important: Including no template control samples (NTCs) in the experimental design will provide information about presence of any DNA contaminants.

DNA purification for detecting microbial DNA targets

Purification of microbial genomic DNA from highly diverse sample material requires the use of dedicated extraction chemistries that are optimized for handling its specific contaminants. Purity of nucleic acid templates is important for dPCR because contaminants can interfere with fluorescence detection. QIAGEN® offers a complete range of nucleic acid purification systems that are dedicated for the isolation of bacterial, fungal, and viral DNA and RNA from a variety of biological samples and that provide pure, high-quality templates for PCR and RT-PCR. These include QIAprep® Kits for purification of plasmid DNA, QIAamp® and DNeasy® Kits for purification of genomic DNA, and AllPrep® Power Kits with inhibitor removal technology. A selection of recommended purification kits is listed in Table 10 on the next page. Details about QIAGEN kits for nucleic acid purification can be found at **qiagen.com/dna-purification/microbial-dna/**.

Table 10. Recommended nucleic acid purification kits for use with QIAGEN's dPCR Microbial DNA Detection Assays

Sample material	Purification kit	Cat. no.
Stool	QIAamp PowerFecal Pro DNA Kit	51804
	QIAsymphony PowerFecal Pro DNA Kit	938036
	RNeasy PowerFecal Pro	78404
	AllPrep PowerFecal Pro DNA/RNA Kit	80254
Blood	QIAamp UCP PurePathogen Blood Kit	50112
	QIAamp DNA Blood Mini Kit	51104
Cervical swab in transport media	QIAamp MinElute Media Kit	57414
Blood culture, bronchoalveolar lavage, various dentine, cervical swab, isolated bacterial colony, sputum, saliva, swabs	QIAamp UCP Pathogen Mini Kit	50214
	QIAamp UCP DNA Micro Kit	56204
	QIAamp DNA Microbiome Kit	51704
Plasma, serum, cell-free body fluids	QIAamp MinElute Virus Kit	57704
Wastewater	AllPrep PowerViral DNA/RNA Kit	28000–50
	RNeasy PowerFecal Pro Kit	78404

DNA purification for detecting mutations

The kits listed below are recommended for genomic DNA purification from various types of starting material for use with QIAGEN's dPCR LNA Mutation Assays (Table 11 on the facing page). DNA purification can be carried out according to the kit instructions.

Table 11. Recommended DNA purification kits for use with QIAGEN's dPCR LNA Mutation Assays

Starting material	Purification method	Cat. no.
Fresh or frozen tissues, cultured cells	QIAamp DNA Mini Kits	51304 and 51306
	DNeasy Blood & Tissue Kits	69506, 69504, 69582, and 69581
	EZ1 DNA Investigator Kit	952034
	EZ1 DNA Tissue Kit	953034
	Blood & Cell Culture DNA Mini, Midi, or Maxi Kit	13323, 13343, or 13362, respectively
	Flexigene DNA Kit	51206
	PAXgene Tissue DNA Kit	767134
FFPE tissues	QIAamp DNA FFPE Tissue Kit	56404
	GeneRead DNA FFPE Kit	180134
cfDNA from human plasma or serum	QIAamp DNA Blood Mini or Maxi Kits	51106, or 51194 and 51192, respectively
	PAXgene Blood DNA Kit	761133
	QIAamp ccfDNA/RNA Kit	55184
	EZ1 ccfDNA Mini or Midi Kit	954134 or 954154, respectively
	QIAamp MinElute ccfDNA Midi and Mini Kits	55284 and 55204, respectively
Other biological samples	Contact QIAGEN Technical Service	

Important: Do not use DEPC-treated water.

Important: Presence of RNA in the sample preparations will result in inaccurate DNA concentration measurements. Removing of RNA contamination using RNase A digest is highly recommended.

Important: Including NTCs in the experimental design will provide information about presence of any DNA contaminants.

DNA quality

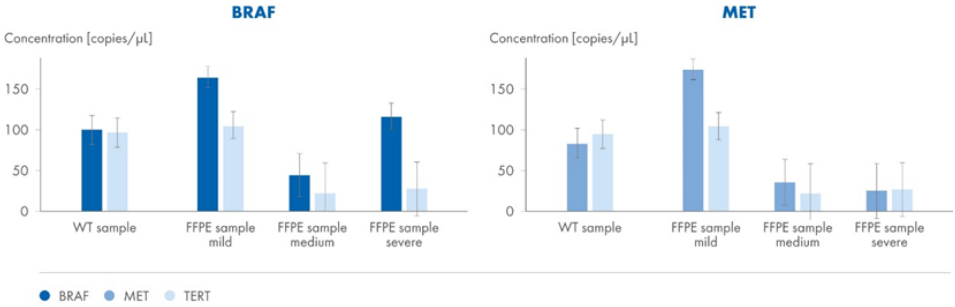
All DNA samples used in reaction mixes should show similar quality and quantity, which can be easily assessed using ultraviolet (UV) spectrophotometry.

DNA samples measured with a UV spectrophotometer should meet the following criteria:

- Concentration determined by A_{260} should be >10 µg/mL.
- A_{260}/A_{230} ratio should be greater than 1.7.
- A_{260}/A_{280} ratio should be greater than 1.8.

Because water is not buffered, the pH and the resulting A_{260}/A_{230} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{230} ratio and reduced sensitivity to protein contamination. For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. For DNA samples that are diluted in 10 mM Tris·Cl, pH 8.0, an absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to a DNA concentration of 50 ug/µL.

Quality-compromised input material, such as DNA samples extracted from FFPE samples with varying degrees of crosslinking and fragmentation, may result in suboptimal quantification (Figure 5 on the facing page) due to possible fragmentation of the genomic region that harbors the gene of interest or target of interest.



	CN/genome	
Sample	**BRAF**	**MET**
WT	2	2
FFPE mild	2.99	3.82
FFPE medium	3.79	3.69
FFPE severe	8.24	2.07

Figure 5. Use of DNA templates with compromised quality (here, fragmented FFPE material) might result in inaccurate target detection. Examples given here are target assays BRAF and MET, which were tested using genomic DNA extracted from FFPE tissue material with varying levels of fragmentation and formalin damage (mild, medium, and severe). With increasing levels of fixation and fragmentation, copy number per genome of both BRAF and MET target assays changed drastically. These results indicate that copy number determination is inaccurate and inconsistent in "severely" fragmented and formalin-compromised FFPE material, when compared to FFPE material with "mild" and "medium" levels of fragmentation and fixation.

Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Genomic DNA isolation kit (refer to "Purification of DNA" on page 27 for DNA purification kit recommendations)
- QIAcuity Digital PCR Instrument (refer to Table 1 on page 4 for the appropriate dPCR cyclers)
- QIAcuity dPCR Nanoplates (refer to Table 1 on page 4 for the appropriate nanoplates)
- QIAcuity High Multiplex Probe PCR Kit
- Multichannel pipettor
- Nuclease-free pipette tips and tubes

Important Notes

For accurate and reproducible dPCR results, it is essential to avoid contamination of the assay with foreign DNA, especially PCR products from previously run nanoplates. The most common sources of DNA contamination are the products of previous experiments and highly concentrated template dilutions.

To maintain a working environment free of DNA contamination, we recommend the following precautions:

- Wear gloves throughout the procedure. Use only fresh PCR-grade labware (tips and tubes).
- Use sterile pipette tips with filters.
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents.
- Physically separate the workspaces used for dPCR setup and post-dPCR processing operations. Decontaminate your dPCR workspace and labware (pipette, tube racks, etc.) with UV light before each new use to render any contaminated DNA ineffective in dPCR through the formation of thymidine dimers or with 10% bleach to chemically inactivate and degrade any DNA.
- Do not open any previously run and stored dPCR nanoplate. Removing the sealer foil from dPCR nanoplate releases dPCR product DNA into the air where it can contaminate the results of future experiments. In the event of contamination ensure that any affected labware and bench surfaces are decontaminated.
- Do not remove the dPCR nanoplate from its protective sealed bag until immediately before use.

Protocol: Detecting One Target per Channel in up to 8 Channels

This protocol is optimized for the detection of 1 target per channel in up to 8 channels in a single reaction using the QIAcuity High Multiplex Probe PCR Kit (cat. nos. 250133, 250134) with the QIAcuity digital PCR (dPCR) instrument.

Important points before starting

- The QIAcuity High Multiplex Probe PCR Kit requires the QIAcuity Software Suite version 3.0 or higher.
- Refer to the *QIAcuity User Manual* and *QIAcuity User Manual Extension* for guidance on assay design and experimental setup for the QIAcuity platform.
- To create a custom crosstalk matrix for a given multiplex setup, singleplex reactions for each of the multiplex targets and a reaction containing no assays must be run. Refer to the technical note "Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity Software Suite 3.0 and 3.1" for more details.

Things to do before starting

- Determine DNA concentration and purity by preparing dilutions and measuring absorbance in 10 mM Tris, pH 8.0 buffer. For best results, the concentration measured at A_{260} should be greater than 10 ng/µL DNA, and the A_{260}/A_{280} ratio should be greater than 1.8.
- Thaw genomic DNA and QIAcuity High Multiplex Probe Mastermix on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex, then quick spin.

Template DNA Digestion

- Before partitioning, DNA samples with an average length ≥20 kb should be digested. This ensures accurate and precise quantification. Restriction digestion is not required for highly fragmented DNA (e.g., FFPE DNA or circulating DNA).
- The restriction enzymes in Table 12 are validated to digest template DNA in 10 min at RT in QIAcuity High Multiplex Probe PCR Master Mix without impairing the subsequent PCR amplification. For assay- specific restriction enzyme compatibility, please go to **geneglobe.qiagen.com** or refer to the product data sheet that is provided with each assay.

Table 12. List of recommended enzymes

6-Cutter restriction enzymes		4-Cutter restriction enzymes	
*EcoR*I	0.25 U/µL EcoRI-HF®, NEB* 0.025 U/µL Anza™ 11 EcoRI, TFS†	*Alu*I	0.025 U/µL AluI, NEB* 0.025 U/µL Anza 44 AluI, TFS
*Pvu*II	0.025 U/µL PvuII, NEB 0.025 U/µL Anza 52 PvuII, TFS	*CviQ*I	0.025 U/µL CviQI, NEB 0.025 U/µL Csp6I (CviQI), TFS
*Xba*I	0.025 U/µL Anza 12 XbaI, TFS	*Hae*III	0.025 U/µL BsuRI (HaeIII), TFS

* NEB, New England Biolabs † TFS, Thermo Fisher Scientific

Important: To ensure that your DNA samples are fully digested, the particular versions of the restriction enzymes from the indicated suppliers must be used.

Procedure

Reaction setup

1. Thaw the 4x QIAcuity High Multiplex Probe PCR Master Mix, template, primers, probes, and RNase-Free Water. Vigorously mix the QIAcuity High Multiplex Probe PCR Master

Mix and the individual solutions. Centrifuge the tubes briefly to settle the liquids.

2. Prepare a master mix according to Table 13 for the desired Nanoplate format.
3. Vortex the reaction mix thoroughly. Dispense appropriate volumes of the reaction mix into the wells of a standard 96-well PCR pre-plate.

   Note: The pre-plate may be assembled at room temperature.

4. Add template DNA to wells containing the reaction mix. Thoroughly mix the template DNA with the reaction mix by pipetting up and down.

Table 13. Preparing reaction mix for detecting up to 8 targets per reaction (one target per channel in up to 8 channels)

	Volume per reaction		
Component	**Nanoplate 8.5k (24-well and 96-well)**	**Nanoplate 26k (8-well and 24-well)**	**Final concentration**
4x QIAcuity High Multiplex Probe PCR Master Mix	3 µL	10 µL	1x
20x Primer–probe mix 1–8*	0.6 µL (each)	2 µL	variable†
Restriction Enzyme (optional)	up to 1 µL	up to 1 µL	0.025–0.25 U/µL
RNase-Free Water	variable	variable	–
Template DNA (added at step 4)‡	variable	variable	–
Total reaction volume	**12 µL**	**40 µL**	–

* For dye recommendations, see the *QIAcuity User Manual* or the *QIAcuity User Manual Extension*.

† For multiplex reactions detecting a single target in any of the six standard QIAcuity channels (Green, Yellow, Orange, Red, Crimson, and Far Red), final assay concentrations of 0.8 µM forward primer, 0.8 µM reverse primer, and 0.4 µM probe are recommended. When using long Stokes-shift dyes, variable assay concentrations are required. Refer to the section "Assay concentration guidelines for detecting one target per channel in up to eight channels" for more details.

‡ Appropriate template amount depends on various parameters.

dPCR protocol for all QIAcuity instruments

1. Transfer the contents of each well in the pre-plate to the wells of a Nanoplate.
2. Seal the Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.
3. Program the dPCR run in the QIAcuity Software Suite or at the QIAcuity instrument. After creating a new plate, move first to the Priming tab under the dPCR parameters section. Select the "QIAGEN Priming Profile Probe (RT-) PCR" priming profile.
4. Move next to the Cycling tab under the dPCR parameters section next and program the dPCR cycling according to Table 14 below.
5. Move to the Imaging tab under the dPCR parameters section next and activate the "Enable High-Multiplexing-Reference channel" toggle as well as the required channels. Imaging settings should be set to the default values.

   Note: If the "Enable High-Multiplexing-Reference channel" toggle is NOT activated, NO valid wells will appear during analysis. For best results, signal in each channel should not exceed 100 RFUs. Signal at or below 100 RFUs minimizes cross talk between channels, which in turn improves data quality. As each assay setup is unique, imaging settings may need to be adjusted.
6. Place the Nanoplate into the QIAcuity instrument and start the dPCR program.

Table 14. QIAcuity dPCR cycling program

Step	Time	Temperature
Initial denaturation	2 min	95°C
2-step cycling (40 cycles)	–	–
Denaturation	15 s	95°C
Combined annealing/extension	30 s	60°C*

* Temperature during annealing/extension and number of cycles might vary depending on assay type.

Data analysis

1. To set up a plate layout according to the experimental design, open the QIAcuity Software Suite and define the reaction mixes, samples, and controls. The plate layout can be defined before or after the nanoplate run.

   Note: Refer to the QIAcuity User Manual for details on setting up the plate layout.

2. After the nanoplate run, the raw data is automatically sent to the QIAcuity Software suite.

3. If custom cross talk compensation is to be applied to the data, generate a custom cross talk matrix using reactions on the plate or import an existing custom cross talk matrix.

   Note: The custom cross talk matrix configurator can be accessed via "Create CXTM" in the Analysis menu or in the Plate Overview. Refer to the technical note "Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity Software Suite 3.0 and 3.1" for more details on how to create a custom cross talk matrix.

   Note: If importing a custom cross talk matrix from an existing template, all of the conditions between the two must be the same: assays used, assay concentrations, and imaging settings. If any one of the parameters between the 2 runs is different, custom cross talk compensation will not be correct.

4. For data analysis, open the QIAcuity Software Suite and select the individual nanoplate for the analysis in Plate Overview of the software suite.

   Note: Refer to the *QIAcuity User Manual Extension: QIAcuity Application Guide* or the *QIAcuity User Manual* for details on how to analyze data for a given application.

Protocol: Detecting up to 12 Targets in 6 Channels with Amplitude-based Multiplexing

This protocol is optimized for the detection of 2 targets per channel in up to 6 channels with amplitude-based multiplexing in a single reaction using the QIAcuity High Multiplex Probe PCR Kit (cat. nos. 250133, 250134) with the QIAcuity digital PCR (dPCR) instrument.

Important points before starting

- Amplitude-based multiplexing with the QIAcuity High Multiplex Probe PCR Kit requires the QIAcuity Software Suite version 3.1 or higher.
- Refer to the *QIAcuity User Manual* and *QIAcuity User Manual Extension* for guidance on assay design and experimental setup for the QIAcuity platform.
- Amplitude-based multiplexing in the hybrid channels is not recommended.
- As mutation detection assays typically do not generate sharp bands of signal, amplitude-based multiplexing for mutation detection is not recommended.
- To create a custom crosstalk matrix for an amplitude-based multiplex setup, duplex reactions containing both assays for a given channel and a reaction containing no assays must be run. Refer to the technical note "Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity Software Suite 3.0 and 3.1" for more details.

Things to do before starting

- Determine DNA concentration and purity by preparing dilutions and measuring absorbance in 10 mM Tris, pH 8.0 buffer. For best results, the concentration measured at A_{260} should be greater than 10 ng/µL DNA, and the A_{260}/A_{230} ratio should be greater than 1.8.

- Thaw genomic DNA and QIAcuity High Multiplex Probe Mastermix on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex, then quick spin.
- Evaluate assays in singleplex and amplitude-based duplex reactions before proceeding with a twelve-plex reaction.

Template DNA Digestion

- Before partitioning, DNA samples with an average length ≥20 kb should be digested. This ensures accurate and precise quantification. Restriction digestion is not required for highly fragmented DNA (e.g., FFPE DNA or circulating DNA).
- The restriction enzymes in Table 15 below are validated to digest template DNA in 10 min at room temperature in QIAcuity High Multiplex Probe PCR Master Mix without impairing the subsequent PCR amplification. For assay-specific restriction enzyme compatibility, please go to **geneglobe.qiagen.com** or refer to the product data sheet provided with each assay.

Table 15. List of recommended enzymes

6-Cutter restriction enzymes		4-Cutter restriction enzymes	
*EcoR*I	0.25 U/µL EcoRI-HF®, NEB* 0.025 U/µL Anza™ 11 EcoRI, TFS†	*Alu*I	0.025 U/µL AluI, NEB* 0.025 U/µL Anza 44 AluI, TFS
*Pvu*II	0.025 U/µL PvuII, NEB 0.025 U/µL Anza 52 PvuII, TFS	*CviQ*I	0.025 U/µL CviQI, NEB 0.025 U/µL Csp6I (CviQI), TFS
*Xba*I	0.025 U/µL Anza 12 XbaI, TFS	*Hae*III	0.025 U/µL BsuRI (HaeIII), TFS

* NEB, New England Biolabs † TFS, Thermo Fisher Scientific

Important: To ensure that your DNA samples are fully digested, the particular versions of the restriction enzymes from the indicated suppliers must be used.

Procedure

Reaction setup

1. Thaw the 4x QIAcuity High Multiplex Probe PCR Master Mix, template, primers, probes, and RNase-Free Water. Vigorously mix the QIAcuity High Multiplex Probe PCR Master Mix and the individual solutions. Centrifuge the tubes briefly to settle the liquids.
2. Prepare a master mix according to Table 16 on the next page for the desired Nanoplate format.
3. Vortex the reaction mix thoroughly. Dispense appropriate volumes of the reaction mix into the wells of a standard 96-well PCR pre-plate.

   Note: The pre-plate may be assembled at room temperature.
4. Add template DNA to wells containing the reaction mix. Thoroughly mix the template DNA with the reaction mix by pipetting up and down.

Table 16. Preparing reaction mix for detecting up to 12 targets per reaction with amplitude-based multiplexing

Component	Volume per reaction		Final concentration
	Nanoplate 8.5k (24-well and 96-well)	Nanoplate 26k (8-well and 24-well)	
4x QIAcuity High Multiplex Probe PCR Master Mix	3 µL	10 µL	1x
20x Primer–probe mix 1–12* (for multiplex)	variable	variable	variable†
Restriction Enzyme (optional)	up to 1 µL	up to 1 µL	0.025–0.25 U/µL
RNase-Free Water	variable	variable	–
Template DNA (added at step 4)‡	variable	variable	–
Total reaction volume	**12 µL**	**40 µL**	–

* For dye recommendations, see the *QIAcuity User Manual* or the *QIAcuity User Manual Extension*.

† For multiplex reactions that employ amplitude-based multiplexing, variable assay concentrations are required. Refer to the "Principle and procedure" section on page 7 and Appendix B on page 56 for technical guidance on setting up amplitude-based multiplex reactions.

‡ Appropriate template amount depends on various parameters.

dPCR protocol for all QIAcuity instruments

1. Transfer the contents of each well in the pre-plate to the wells of a Nanoplate.
2. Seal the Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.
3. Program the dPCR run in the QIAcuity Software Suite or at the QIAcuity instrument. After creating a new plate, move first to the Priming tab under the dPCR parameters section. Select the "QIAGEN Priming Profile Probe (RT-) PCR" priming profile.

4. Move next to the Cycling tab under the dPCR parameters section next and program the dPCR cycling according to Table 17 below.
5. Move to the Imaging tab under the dPCR parameters section next and activate the "Enable High-Multiplexing-Reference channel" toggle as well as the required channels. Imaging settings should be set to the default values.

   Note: If the "Enable High-Multiplexing-Reference channel" toggle is NOT activated, NO valid wells will appear during analysis. Refer to Figure 6 on page 57 for recommendations on the maximum RFU levels for each channel when performing amplitude-based multiplexing. Since each assay setup is unique, you may need to adjust the imaging settings accordingly.
6. Place the Nanoplate into the QIAcuity instrument and start the dPCR program.

Table 17. QIAcuity dPCR cycling program

Step	Time	Temperature
Initial denaturation	2 min	95°C
2-step cycling (40 cycles)	–	–
Denaturation	15 s	95°C
Combined annealing/extension	30 s	60°C*

* Temperature during annealing/extension and number of cycles might vary depending on assay type.

Data analysis

1. To set up a plate layout according to the experimental design, open the QIAcuity Software Suite and define the reaction mixes, samples, and controls. The plate layout can be defined before or after the nanoplate run.

   Note: Refer to the *QIAcuity User Manual* for details on setting up the plate layout.

2. After the nanoplate run, the raw data is automatically sent to the QIAcuity Software suite.
3. If custom cross talk compensation is to be applied to the data, generate a custom cross talk matrix using reactions on the plate or import an existing custom cross talk matrix.

   Note: The custom cross talk matrix configurator can be accessed via "Create CXTM" in the Analysis menu or in the Plate Overview. Refer to the technical note "Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity Software Suite 3.0 and 3.1" for more details on how to create a custom cross talk matrix.

   Note: If importing a custom cross talk matrix from an existing template, all of the conditions between the 2 must be the same: assays used, assay concentrations, and imaging settings. If any one of the parameters between the 2 runs is different, custom cross talk compensation will not be correct.

4. To perform amplitude-based multiplexing analysis, tick the "Amplitude Multiplexing" option in the 1D Scatterplot view.

   Note: The Amplitude Multiplexing analysis feature may be activated for just one channel, a selection of channels, or for all channels. Similarly, the Amplitude Multiplexing analysis feature may be activated for just one well, a selection of wells, or for the entire plate. Depending on target concentration and the separation of positive clusters in the 1D scatterplots, users may have to manually set thresholds. To aid in thresholding, a positive control that generates three clusters of positive signal in each channel should be run.

   Note: Secondary analysis functions within the QIAcuity Software Suite (e.g., Copy Number Variation analysis) are currently unavailable for use with amplitude-based multiplexing.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center (**www.qiagen.com/FAQ/FAQList.aspx**). The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
No valid wells were detected	
1. The High-Multiplexing-Reference channel was not selected when defining the imaging settings	The QIAcuity High Multiplex Probe PCR Kit requires the "Enabling High-Multiplexing Reference channel" option to be activated in the Imaging settings. Otherwise, the passive reference dye in will not be recognized.
Weak or no signal	
2. The selected fluorescence channel for dPCR data analysis does not comply with the protocol	For data analysis, select the channel corresponding to the dye used for the assay.
3. Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile programmed on the instrument with the cycling programs outlined here in this handbook. If assays come with specific cycling guidelines that differ from those outlined in this handbook (e.g. different annealing temperatures), use those instead.
4. PCR extension time too short	Use the extension time specified in the protocol.
5. PCR was inhibited	Use the recommended DNA isolation methods and closely follow the manufacturer's instructions. QIAGEN offers dedicated sample preparation kits developed to complement the various applications for use with the QIAcuity High Multiplex Probe PCR Kit. Refer to the section "Purification of DNA" on page 27 for more details.

		Comments and suggestions
6.	Incorrect configuration of the PCR	Ensure that reactions were set up according to the reaction mix preparation described in the reaction setup on page 37 or on page 43. Repeat the dPCR run if necessary.
7.	The storage conditions for one or more kit components did not comply with the instructions given in "Shipping and Storage".	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
8.	Insufficient starting template	Increase the amount of template genomic DNA.
9.	Restriction enzyme cuts within amplicon	If restriction enzymes cut within the PCR amplicon sequence, little or no signal will be generated. Confirm that the correct restriction enzyme was chosen for the experiment and repeat. Alternatively, use another restriction enzyme to perform DNA digestion.
Signals present for the negative template control (NTC copies/µL > 0)		
1.	Contamination occurred during PCR setup	Repeat the PCR with new reagents. Make sure to pipette the positive controls last. Make sure that workspace and instruments are decontaminated at regular intervals.
2.	Contamination occurred during extraction	Repeat the extraction and PCR of the sample to be tested using new reagents. Make sure that workspace and instruments are decontaminated at regular intervals.
3.	Assay Design is sub-optimal	If possible, choose another assay design for the target of interest and repeat the experiment.
Underquantification of target		
1.	Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile programmed on the instrument with the cycling programs outlined here in this handbook. If assays come with specific cycling guidelines that differ from those outlined in this handbook (e.g. different annealing temperatures), use those instead.
2.	PCR extension time too short	Use the extension time specified in the protocols outlined in this handbook.

		Comments and suggestions
3.	PCR was inhibited	Use the recommended DNA isolation methods and closely follow the manufacturer's instructions. QIAGEN offers dedicated sample preparation kits developed to complement the various applications for use with the QIAcuity High Multiplex Probe PCR Kit. Refer to the section "Purification of DNA" on page 27 for more details.
4.	Incorrect configuration of the PCR	Ensure that reactions were set up according to the reaction mix preparation described in the reaction setup on page 37 or on page 43. Repeat the dPCR run, if necessary.
5.	The storage conditions for one or more kit components did not comply with the instructions given in "Shipping and Storage".	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
6.	Structure of locus containing PCR targets is challenging for PCR reaction	For some loci in the genome, the structure of the DNA can prove challenging for PCR reactions. Without proper fragmentation, challenging DNA structures can lead to the underquantification of DNA targets contained within them. If restriction enzyme digestion was performed, try digestion with another restriction enzyme. If possible, use a compatible 4-cutter restriction enzyme to fragment the sample DNA, since it will cut the genome more frequently than a 6-cutter restriction enzyme.
7.	Assay design is sub-optimal	If possible, choose another assay design for the target of interest and repeat the experiment.
8.	Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile programmed on the instrument with the cycling programs outlined here in this handbook. If assays come with specific cycling guidelines that differ from those outlined in this handbook (e.g. different annealing temperatures), use those instead.
Overquantification of target		
1.	Assay design is sub-optimal	If possible, choose another assay design for the target of interest and repeat the experiment.
Suboptimal 1D Scatterplots in multiplex reactions detecting one target per channel in up to eight channels (e.g. rain, low fluorescence, fluorescence saturation, cross talk)		
1.	Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile programmed on the instrument with the cycling programs outlined here in this handbook. If assays come with specific cycling guidelines that differ from those outlined in this handbook (e.g. different annealing temperatures), use those instead.

		Comments and suggestions
2.	PCR extension time too short	Use the extension time specified in the protocol.
3.	PCR was inhibited	Use the recommended DNA isolation method and closely follow the manufacturer's instructions. QIAGEN offers dedicated sample preparation kits developed to complement a variety of applications.
4.	Incorrect configuration of the PCR	Ensure that reactions were set up according to the reaction mix preparation described in the reaction setup on page 37 or on page 43. Repeat the dPCR run, if necessary.
5.	The storage conditions for one or more kit components did not comply with the instructions given in "Shipping and Storage".	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
6.	Structure of locus containing PCR targets is challenging for PCR reaction	For some loci in the genome, the structure of the DNA can prove challenging for PCR reactions. Without proper fragmentation, challenging DNA structures can lead to rainy 1D scatterplots or positive amplification clusters with low fluorescence. If restriction enzyme digestion was performed, try digestion with another restriction enzyme. If possible, use a compatible 4-cutter restriction enzyme to fragment the sample DNA, since it will cut the genome more frequently than a 6-cutter restriction enzyme.
7.	When using long Stokes-shift dyes, assay conditions may need to be optimized.	Refer to Appendix A for guidance on using long Stokes-shift dyes.
8.	When using long Stokes-shift dyes, the default custom cross talk compensation must be used.	The default cross talk compensation is not applied when hybrid channels (e.g., Green/Yellow, Orange/Red) are used. Refer to the technical note "Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity Software Suite 3.0 and 3.1" for more details on how to create a custom cross talk matrix

		Comments and suggestions
9.	Assays were reconstituted incorrectly (too dilute)	If assays are reconstituted incorrectly, such that they are too dilute, the end fluorescence in the 1D Scatterplots will be weaker than normal. If customers know by which factor the assay was incorrectly reconstituted, they can compensate for this error during reaction setup by adding the appropriate amount more of the assay. If the assay was diluted such that it no longer fits into the reaction setup, customers may evaporate the assay in a vacuum concentrator (e.g. SpeedVac™). Following evaporation, the assay may be reconstituted again using the correct volume of nuclease-free water indicated in its accompanying product data sheet.
10.	Assay was reconstituted incorrectly (too concentrated)	If assays are reconstituted incorrectly, such that they are too concentrated, the end fluorescence in the 1D scatterplots will be much stronger than normal, potentially reaching saturation. If customers know by which factor the assay was incorrectly reconstituted, they can compensate for this error during reaction setup by adding less of the assay to their reaction mixes.

Suboptimal 1D scatterplots in amplitude-based multiplex reactions detecting up to twelve targets (e.g., rain, low fluorescence, fluorescence saturation, cross talk)

1.	Incorrect programming of the QIAcuity dPCR instrument	Compare the temperature profile programmed on the instrument with the cycling programs outlined here in this handbook. If assays come with specific cycling guidelines that differ from those outlined in this handbook (e.g. different annealing temperatures), use those instead.
2.	PCR extension time too short	Use the extension time specified in the protocol.
3.	PCR was inhibited	Use the recommended DNA isolation method and closely follow the manufacturer's instructions. QIAGEN offers dedicated sample preparation kits developed to complement a variety of applications.
4.	Incorrect configuration of the PCR	Ensure that reactions were set up according to the reaction mix preparation described in the reaction setup on page 37 or on page 43. Repeat the dPCR run, if necessary.
5.	The storage conditions for one or more kit components did not comply with the instructions given in "Shipping and Storage".	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.

		Comments and suggestions
6.	Structure of locus containing PCR targets is challenging for PCR reaction	For some loci in the genome, the structure of the DNA can prove challenging for PCR reactions. Without proper fragmentation, challenging DNA structures can lead to rainy 1D scatterplots or positive amplification clusters with low fluorescence. If restriction enzyme digestion was performed, try digestion with another restriction enzyme. If possible, use a compatible 4-cutter restriction enzyme to fragment the sample DNA, since it will cut the genome more frequently than a 6-cutter restriction enzyme.
7.	Long Stokes-shift dyes were used	Long Stokes-shift dyes are not well suited for amplitude-based multiplexing
8.	Mutation detection assays were used	The signal generated by mutation detection assays is not well suited for amplitude-based multiplexing
9.	Custom cross talk compensation was not used.	While the default cross talk compensation in the QIAcuity Software Suite version 3.1 may suffice for some amplitude-based multiplexing applications, the quality of results greatly benefits from the use of a custom cross talk matrix. Refer to the technical note "Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity Software Suite 3.0 and 3.1" for more details on how to create a custom cross talk matrix
10.	Assay concentrations not optimized to generate three distinct clusters	After screening individual assay performance, the tuning of assay concentrations to generate three distinct clusters of positive signal in 1D scatterplots is the second most critical aspect of amplitude-based multiplexing. Refer to the Appendix B for more details on the impact of titrating assay concentration on the appearance of signal in 1D Scatterplots.
11.	Assays were reconstituted incorrectly (too dilute)	If assays are reconstituted incorrectly, such that they are too dilute, the end fluorescence in the 1D scatterplots will be weaker than normal. If customers know by which factor the assay was incorrectly reconstituted, they can compensate for this error during reaction setup by adding the appropriate amount more of the assay. If the assay was diluted such that it no longer fits into the reaction setup, customers may evaporate the assay in a vacuum concentrator (e.g., SpeedVac™). Following evaporation, the assay may be reconstituted again using the correct volume of nuclease-free water indicated in its accompanying product data sheet.
12.	Assay was reconstituted incorrectly (too concentrated)	If assays are reconstituted incorrectly, such that they are too concentrated, the end fluorescence in the 1D scatterplots will be much stronger than normal, potentially reaching saturation. If customers know by which factor the assay was incorrectly reconstituted, they can compensate for this error during reaction setup by adding less of the assay to their reaction mixes.

	Comments and suggestions
Background signal increases and positive signal becomes less sharp/smeared when reimaging plates a few days after being run	
1. For some assays, residual polymerase activity following PCR cycling may drive changes (over the course of days) in the appearance of signal in 1D Scatterplots in plates	The cause of this phenomenon is residual DNA polymerase activity. To inactivate the polymerase after cycling, users may include a final step of 20 to 30 minutes at 98°C to their dPCR program.

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Appendix A: Guidelines for Using Long Stokes-shift (LSS) Dyes with the QIAcuity High Multiplex Probe PCR Kit

Multiplexing with long Stokes-shift (LSS) dyes requires optimization of assay concentrations and imaging settings

Long Stokes-shift dyes contribute cross talk to multiple channels and are themselves impacted by cross talk from multiple channels. When assays in hybrid channels are yielding poor results (e.g., multiple bands, unexpected shapes in 1D scatterplots, or quantification issues) please review the setup parameters of the corresponding "standard" channels.

For example, if there is an issue with an assay in the Orange/Red channel, adjusting the concentrations of the assays in the Orange or Red channel may be required. As cross talk between channels is the major driver of issues in multiplex reactions with LSS dyes, lowering the concentration of either the Red or Orange assays is a good starting point for a troubleshoot. Additionally, the exposure settings in Orange, Red, or Orange/Red channel may also require adjustment. If too much cross talk between channels is interfering with results, lowering the exposure times for the affected LSS dye and its corresponding standard channels is recommended.

Appendix B: Guidelines for amplitude-based multiplexing with the QIAcuity High Multiplex Probe PCR Kit

Assays that generate sharp signal in 1D Scatterplots are the key to successful amplitude-based multiplexing

While amplitude- based multiplexing may seem intimidating at first, it is actually a straightforward method if one major requirement is fulfilled: positive signal in the 1D scatterplot for a given assay needs to be sharp. When detecting 2 targets in the same channel with amplitude-based multiplexing, both assays must generate sharp bands of positive signal in the 1D scatterplots. While assays that generate "rain" or diffuse bands of positive signal in 1D scatterplots can be used to accurately quantify targets on their own, they are not well suited for amplitude-based multiplexing. When 2 assays generate clear signal on their own, they can be easily combined in amplitude-based multiplexing reactions (Figure 6). Therefore, users must screen the quality of individual assays before proceeding with amplitude-based multiplexing experiments.

Figure 6. Assays must generate clear bands of signal in 1D Scatterplots for amplitude based multiplexing. Assays detecting 2 different targets generate sharp bands of positive signal in the Green channel when run in singleplex. Target A was amplified in singleplex in well B1, while the target B was amplified in singleplex in well B2. When the assays for targets A and B are combined in an amplitude-based multiplex reaction, 3 sharp clusters of positive signal are generated. Multiple thresholds can be clearly set between these clusters. The lower cluster corresponds to partitions positive for Target B only, while the middle cluster corresponds to partitions positive for Target A only. The uppermost cluster corresponds to partitions positive for both Target A and B.

Amplitude multiplexing with QIAcuity dPCR DNA Microbial Detection Assays requires assay pre-screening

QIAcuity dPCR DNA Microbial Detection Assays detect a range of organisms, including bacteria, viruses, and fungi. Therefore, a general guideline regarding the compatibility of assays from this portfolio with amplitude-based multiplexing cannot be provided.

As mentioned previously, the most critical factor to successful amplitude-based multiplexing is the use of assays that produce sharp bands of positive signal in the 1D scatterplots. For some dPCR DNA Microbial Detection Assays, such as bacterial assays targeting conserved genomic regions (e.g., the 16S gene in bacteria), the sharpness of the 1D scatterplots may not be sufficient for amplitude-based multiplexing. Furthermore, when combining assays designed around the same conserved region, the oligonucleotides in the assays are apt to cross-react. Therefore, users must test their assays individually and in combination before proceeding with amplitude-based multiplexing.

Many microbial assays are used to survey environmental samples. The naturally occurring genetic diversity for a particular microbial species in such samples may produce positive signal in 1D scatterplots that is particularly rainy, diffuse, or forms multiple clusters. The extent to which this occurs is apt to differ from sample to sample. In scenarios where such 1D Scatterplots are generated, amplitude-based multiplexing will not be possible.

For some dPCR DNA Microbial Detection Assays, datasheets on **geneglobe.com** provide 1D scatterplots generated with the QIAcuity Probe PCR Kit. These can be used to estimate whether or not an assay is a candidate for amplitude based multiplexing.

Assay concentrations may require fine tuning to ensure clear separation between signal in 1D Scatterplots

When detecting 2 targets in the same channel with amplitude-based multiplexing, the 2 assays must generate clear bands of positive signal in 1D Scatterplots. The handbook section "Assay concentration guidelines for amplitude-based multiplexing" on page 15 provides a starting point for choosing assay concentrations for amplitude based multiplexing. However, users may need to finely adjust the concentrations for their particular assays. To demonstrate the impact of assay concentrations on the appearance of signal in 1D scatterplots, the results of 2 assay titrations in the Green channel are shown in Figure 6.

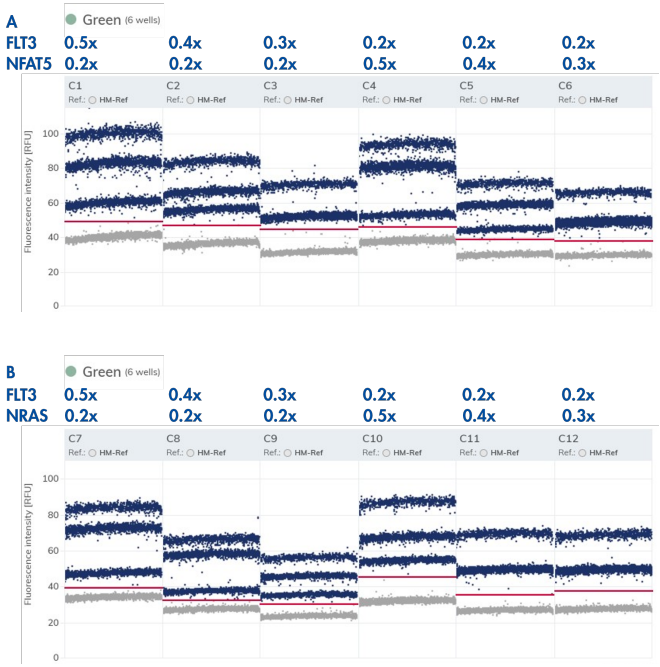


Figure 7. Adjusting the concentrations of individual assays alters the pattern of signal in amplitude-based multiplexing reactions. Two pairs of QIAcuity dPCR CNV Probe Assays, all labeled with FAM, were used to amplify human genomic DNA at a final concentration of 1000 copies/µL in amplitude-based multiplex reactions. One assay pair comprised the **(A)** FLT3 and NFAT5 assays, while the second pair comprised the **(B)** FLT3 and NRAS assays. When the final concentration of the individual assays was modulated between reactions, the amplitudes of the positive signal in the 1D Scatterplots changed correspondingly. For some combinations of assay concentrations (e.g., 0.2x FLT3, 0.4x NRAS) it was no longer possible to distinguish the 3 clusters of positive signal that were to be expected when both targets were present at 1000 copies/µL.

Ordering Information

Product	Contents	Cat. no.
QIAcuity High Multiplex Probe PCR Kit (1 mL)	4x QIAcuity High Multiplex Probe PCR Mastermix, QuantiNova Internal Control DNA dPCR, RNase-Free Water	250133
QIAcuity High Multiplex Probe PCR Kit (1 mL)	4x QIAcuity High Multiplex Probe PCR Mastermix, QuantiNova Internal Control DNA dPCR, RNase-Free Water	250134
Accessories		
Nanoplate 8-well 26K	Microfluidic digital PCR plates for 24 samples with up to 26,000 partitions each	250031
Nanoplate 24-well 26K	Microfluidic digital PCR plates for 24 samples with up to 26,000 partitions each	250001
Nanoplate 24-well 8.5K	Microfluidic digital PCR plates for 24 samples with up to 8500 partitions each	250011
Nanoplate 96-well 8.5K	Microfluidic digital PCR plates for 96 samples with up to 8500 partitions each	250021
Nanoplate Seals	11 top seals for the nanoplates	250099
QIAcuity One, 2plex	One-plate digital PCR instrument for detecting up to 2 fluorescent dyes	911001
QIAcuity One, 5plex	One-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911021
QIAcuity Four, 5plex	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911042
QIAcuity Eight, 5plex	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911052
Related Products		
QIAamp DNA Mini Kit (50)	For 50 DNA preps: 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 mL)	51304

Product	Contents	Cat. no.
QIAamp DNA Mini Kit (250)	For 250 DNA preps: 250 QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2 mL)	51306
DNeasy Blood & Tissue Kit (250)	250 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 mL)	69506
DNeasy Blood & Tissue Kit (50)	50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 mL)	69504
DNeasy 96 Blood & Tissue Kit (12)	For 12 x 96 DNA minipreps: 12 DNeasy 96 Plates, Proteinase K, Buffers, S-Blocks, AirPore Tape Sheets, Collection Microtubes (1.2 mL), Elution Microtubes RS, Caps, 96-Well Plate Registers	69582
DNeasy 96 Blood & Tissue Kit (4)	For 4 x 96 DNA minipreps: 4 DNeasy 96 Plates, Proteinase K, Buffers, S-Blocks, AirPore Tape Sheets, Collection Microtubes (1.2 mL), Elution Microtubes RS, Caps, 96-Well Plate Registers	69581
EZ1&2 DNA Investigator Kit (48)	For 48 preps: Reagent Cartridges (DNA Investigator), Disposable Filter-Tips, Disposable Tip-Holders, Sample Tubes (2 mL), Elution Tubes (1.5 mL), Buffer G2, Proteinase K, Carrier RNA	952034
EZ1&2 DNA Tissue Kit (48)	For 48 preps: Reagent Cartridges (Tissue), Disposable Filter-Tips, Disposable Tip-Holders, Sample Tubes (2 mL), Elution Tubes (1.5 mL), Buffer G2, Proteinase K, Carrier RNA	953034
Blood & Cell Culture DNA Mini Kit (25)	25 QIAGEN Genomic-tip 20/G, QIAGEN Protease, Buffers	13323
Blood & Cell Culture DNA Midi Kit (25)	25 QIAGEN Genomic-tip 100/G, QIAGEN Protease, Buffers	13343
Blood & Cell Culture DNA Maxi Kit (10)	10 QIAGEN Genomic-tip 500/G, QIAGEN Protease, Buffers	13362
FlexiGene DNA Kit (250)	For isolation of genomic DNA from whole blood, buffy coat, and cultured cells in a single tube with fast kit preparation	51206

Product	Contents	Cat. no.
PAXgene Tissue DNA Kit (50)	For 50 DNA preps: PAXgene DNA Mini Spin Columns, Processing Tubes, Microcentrifuge Tubes, Carrier RNA, and Buffers; to be used in conjunction with PAXgene Tissue Containers	767134
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute Columns, Proteinase K, Buffers, Collection Tubes (2 mL)	56404
QIAamp DNA FFPE Advanced UNG Kit (50)	For 50 preps: Uracil-N-glycosylase, QIAamp UCP MinElute columns, collection tubes, Deparaffinization Solution, Proteinase K, RNase A, RNase-free water and buffers	56704
QIAamp DNA Blood Mini Kit (250)	For 250 DNA minipreps: 250 QIAamp Mini Spin Columns, QIAGEN Protease, Reagents, Buffers, Collection Tubes (2 mL)	51106
QIAamp DNA Blood Maxi Kit (50)	For 50 DNA maxipreps: 50 QIAamp Maxi Spin Columns, QIAGEN Protease, Buffers, Collection Tubes (50 mL)	51194
QIAamp DNA Blood Maxi Kit (10)	For 10 DNA maxipreps: 10 QIAamp Maxi Spin Columns, QIAGEN Protease, Buffers, Collection Tubes (50 mL)	51192
PAXgene Blood DNA Kit (25)	Processing tubes and buffers for 25 preparations	761133
QIAamp ccfDNA/RNA Kit (50)	For 50 preps: RNeasy Midi and RNeasy MinElute Spin Columns, Collection Tubes (50 mL), Elution Tubes (1.5 mL and 2 mL), RNase-Free Reagents and Buffers	55184
EZ1&2 ccfDNA Kit (48)	For 48 preps (2, 4, or 8 mL sample input volume each): 48 reagent cartridges (EZ1&2 ccfDNA), Magnetic Bead Suspension, Elution Buffer, Large-Volume Tubes (7 mL), Disposable Tip Holders, Disposable Filter-Tips, Elution Tubes (1.5 mL)	954854
QIAamp MinElute ccfDNA Midi Kit (50)	For 50 preps (4 or 5 mL sample input volume each): QIAamp UCP MinElute Columns, QIAGEN Proteinase K, Magnetic Bead Suspension, Buffers, Bead Elution Tubes, and Collection Tubes (1.5 mL and 2 mL)	55284

Product	Contents	Cat. no.
QIAamp MinElute ccfDNA Mini Kit (50)	For 50 preps (1 or 2 mL sample input volume each): QIAamp UCP MinElute Columns, QIAGEN Proteinase K, Magnetic Bead Suspension, Buffers, Bead Elution Tubes, Collection Tubes (1.5 mL and 2 mL)	55204
QIAcube Connect — for fully automated nucleic acid extraction with QIAGEN spin-column kits		
QIAcube Connect*	Instrument, connectivity package, 1-year warranty on parts and labor	Inquire

* All QIAcube Connect instruments are provided with a region-specific connectivity package, including Tablet and equipment necessary to connect to the local network. Further, QIAGEN offers comprehensive instrument service products, including service agreements, installation, introductory training and preventive subscription. Contact your local sales representative to learn about your options.

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.

Document Revision History

Date	Description
02/2025	Initial release

Limited License Agreement for QIAcuity High Multiplex Probe PCR Kit

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