How to set up a higher-order multiplex dPCR assay with the dPCR RNA-Plex Assays and the QIAcuity OneStep High Multiplex PCR Kit

Introduction

Higher-order multiplexing enables the simultaneous detection and quantification of up to 12 targets in a single PCR reaction. Generating more information from each well conserves precious sample, reduces reagent use and hands-on time and minimizes the variation introduced when the same sample is distributed across several singleplex reactions.

In digital PCR (dPCR), higher-order multiplexing can be achieved by combining traditional color-based multiplexing with amplitude multiplexing. Color-based multiplexing assigns targets to different fluorescence channels, while amplitude multiplexing distinguishes two assays within the same channel by adjusting their endpoint fluorescence intensities. This creates separate positive-partition clusters for each target, allowing the software to classify and quantify them independently (Figure 1).

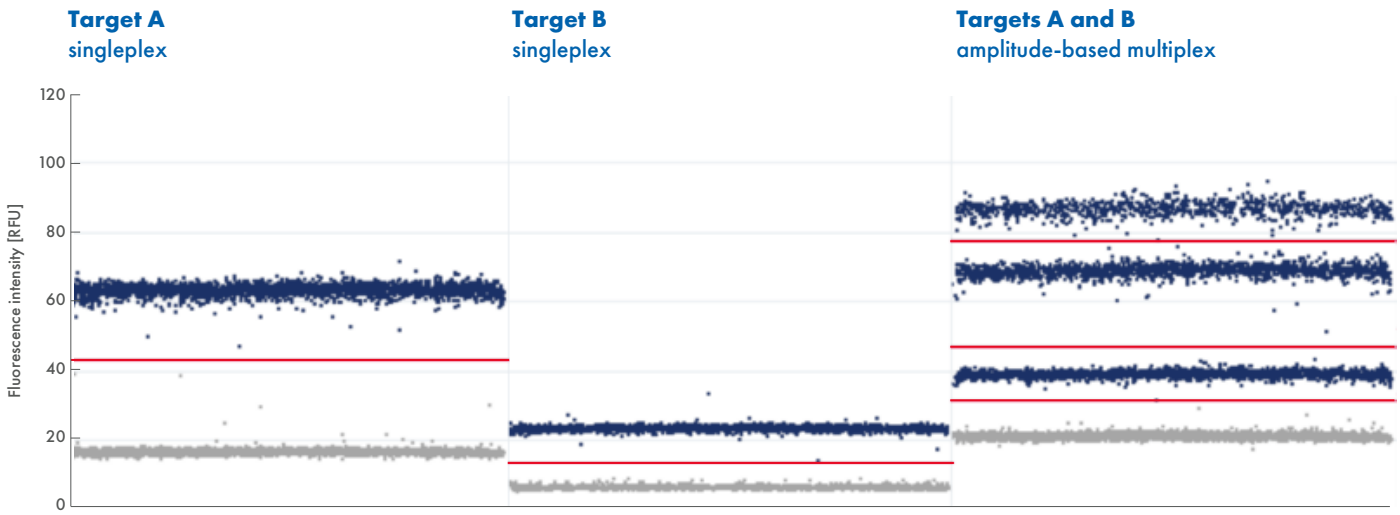


Figure 1. Differentiation of targets by amplitude multiplexing. Example 1D scatterplots for two assays detecting Targets A and B in the Green channel. In the singleplex reactions, the assays for Target A and Target B each generate a single, sharp positive-signal cluster (dark blue). In this example, Target A produces a higher fluorescence amplitude because its assay is used at a higher concentration than Target B. When both assays are combined in an amplitude-based multiplex reaction, three clearly separated positive-signal clusters are generated above the negative-signal cluster (gray). When the reaction is defined as an amplitude multiplex, three thresholds (red lines) separate the negative cluster and the three positive clusters. The lowest positive cluster represents partitions containing Target B only, the middle cluster represents partitions containing Target A only and the highest cluster represents partitions containing both Targets A and B.

Successful higher-order multiplexing depends on robust individual assays, sufficient separation between negative and positive partitions, clearly distinguishable fluorescence amplitudes and minimal interactions or competition among assays. dPCR is particularly well-suited to higher-order multiplexing because the sample is partitioned into thousands of endpoint reactions. Quantification is based on counting positive and negative partitions rather than on amplification curves, while partitioning reduces target competition and supports reliable detection even at varying assay efficiencies.

The following guidelines provide a step-by-step workflow for establishing a higher-order multiplex dPCR assay using the dPCR RNA-Plex Assays (cat. no. 250336, 250337) and the QIAcuity OneStep High Multiplex PCR Kit (cat. nos. 250135, 250136).

The guidelines demonstrate the setup of an amplitude-multiplex reaction that combines 12 separate assays, detected in six channels, with two targets per channel. Also included are examples of well-performing singleplex assays, tips on adjusting endpoint relative fluorescence units (RFUs) when combining two targets per channel, advice on setting up a custom crosstalk matrix and guidance on obtaining and verifying the final assembly of the 12-plex assay.

The same procedure can be applied to establishing multiplexed assays with fewer than 12 targets, either by using fewer colors or by incorporating amplitude multiplexing in only a subset of channels. The guidelines are also applicable to multiplex assays other than the dPCR RNA-Plex assays.

Before you start

- For gene expression analysis, select stable reference genes with expression levels comparable to the targets of interest.
- Prepare suitable positive-control RNA material that contains all targets included in the multiplex assay. Representative internal control RNA, e.g. Human XpressRef Universal Total RNA (cat. no. 338112), may be included if appropriate for the intended sample type or experimental purpose.
- Select targets that would yield reliable detection levels if combined in multiplexed analysis.
- Amplitude multiplexing is compatible with QIAcuity Software Suite Version 3.1 or higher.

Stepwise Guideline

There are three steps in successful amplitude-based multiplexing. First, establish clean, robust singleplex assays. Second, fine-tune the paired assay concentrations until every channel shows three clearly separated positive clusters. Third, generate a custom crosstalk matrix.

Step 1: Screen and select well-performing singleplex assays

Amplitude-based multiplexing is most straightforward to set up and interpret when each assay generates a sharp, well-defined positive band in the 1D scatterplot. Assays with substantial rain or diffuse signals may suffice in singleplex reactions, but are unsuitable for amplitude multiplexing. Therefore, screen all assays individually using the recommended cycling and imaging settings and combine only those fulfilling the following criteria:

- Sharp, well-defined positive clusters in the 1D scatterplot
- Clear separation between negative and positive partitions of >20 relative fluorescence units (RFU)
- Low background signal of negative partitions (ideally <20 RFU)
- Minimal rain
- Expected quantifications in the positive controls and no positive signals in the no-template controls

Replace any poor-performing assay and repeat the evaluation before proceeding to Step 2. For a visual reference of assay profiles that would be considered suitable or unsuitable for higher-order multiplexing, refer to Figure 2.

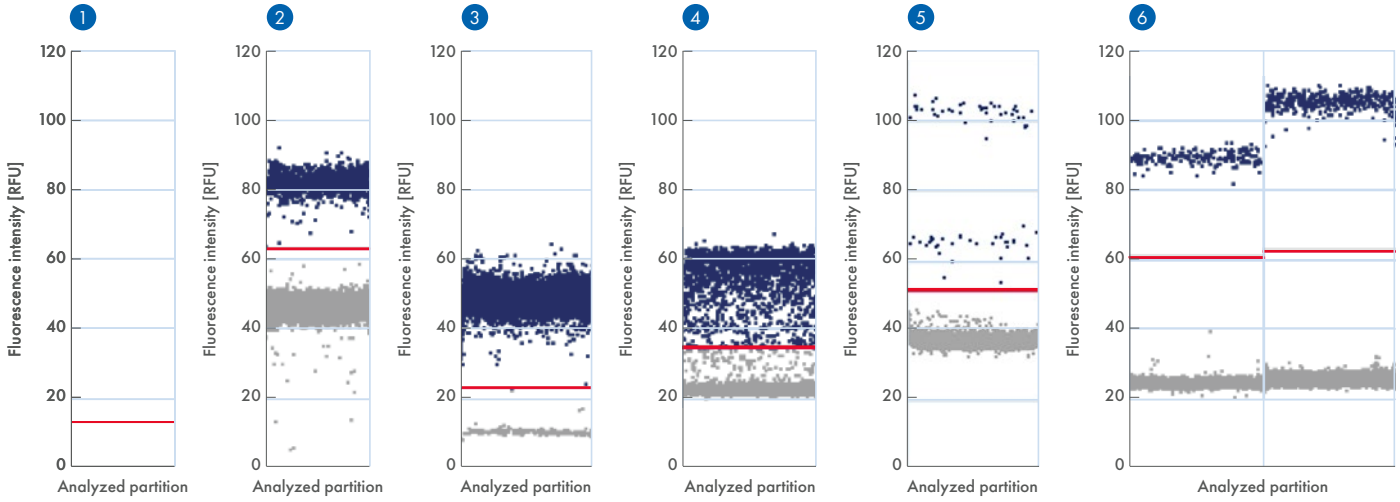


Figure 2. Examples of assay profiles suitable and unsuitable for amplitude multiplexing. Representative 1D scatterplots illustrate assay performance characteristics that limit reliable amplitude multiplexing, including low delta RFU between negative and positive partitions (1), high background fluorescence (2) broad positive clusters (3), substantial rain (4) and secondary signal cluster (5). Two well-performing assays (6) are shown for comparison, each displaying low background, minimal rain and a sharp, clearly separated positive signal cluster.

Step 2: Adjust endpoint RFUs of single assays for amplitude multiplexing

Set assay concentrations: To detect two targets in the same channel, use different assay concentrations to generate clearly separated fluorescence amplitudes. As a starting point, for each assay pair of the same channel, use the assay with the lower RFU in Step 1 at 0.75x and the other at 1.25x of the standard 1x concentration used in Step 1. Run the two assays together in a duplex reaction with 125–1250 copies/µL of each template and review the 1D scatterplot. If any of the three positive clusters overlap or are insufficiently separated, adjust one or both assay concentrations stepwise until three distinct clusters are obtained. For an example of the stepwise concentration adjustments for obtaining three separated positive clusters, see Figure 3.

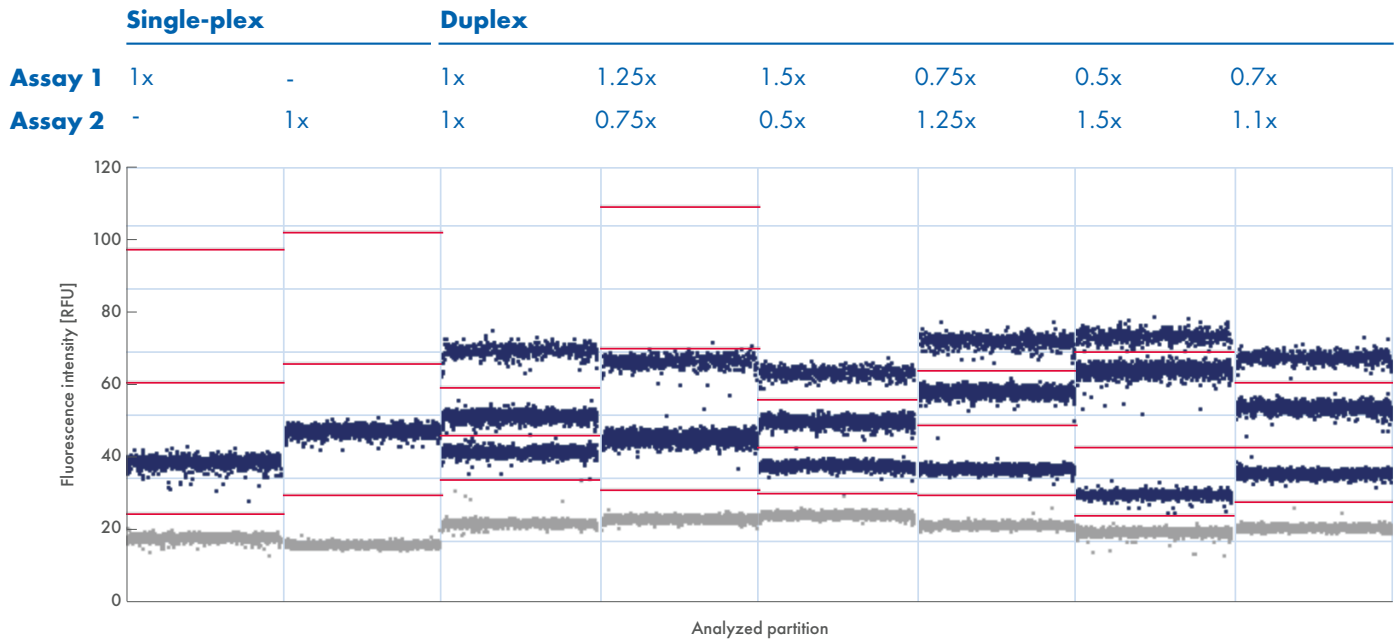


Figure 3. Fine-tuning endpoint RFU levels for amplitude multiplexing. Two assays labeled with FAM dye are shown individually in singleplex and together in duplex at different assay concentrations. Adjusting the assay concentrations shifts the endpoint RFU levels and creates three clearly separated positive clusters representing each target individually and both targets together.

Optimize signal intensity: Check the positive-signal intensity in each channel against the recommended maximum RFU values in Table 1. Pay particular attention to the Yellow, Orange and Red channels, where high signal intensities can increase optical crosstalk. If a signal exceeds the recommended value or cluster separation is poor, reduce the corresponding QIAcuity imaging setting and reimage the plate.

Lower the exposure time for the affected channel stepwise, while keeping the gain unchanged, until the positive signal is below the recommended maximum and remains clearly separated from the negative cluster. As a rule of thumb, reducing the exposure time by 50% results in an approximately 50% lower RFU. Select settings that keep signals within the recommended RFU range while maintaining clear separation from the negative cluster.

Table 1. Guidelines for maximum RFU levels in amplitude-based multiplexing reactions.

Channels	Recommended maximum RFU levels
Green	100 RFU
Yellow	80 RFU
Orange	80 RFU
Red	80 RFU
Crimson	100 RFU
Far Red	100 RFU

Step 3: Generate and apply a custom crosstalk matrix (CXTM)

Crosstalk occurs when fluorescence from one dye is detected in a neighboring optical channel. The extent of this spectral spillover depends on the fluorophore, probe concentration, endpoint signal intensity, reaction chemistry and imaging settings. If crosstalk is not adequately compensated, it can lead to additional bands appearing in the 1D scatterplot, complicating threshold placement and affecting target classification and quantification.

When using amplitude multiplexing, a CXTM is recommended because it measures the fluorescence spillover generated by the actual dyes, assay concentrations, reaction conditions and imaging settings used in the experiment, thereby correcting RFU levels in neighboring channels. Default crosstalk compensation is automatically applied to reactions targeting up to six targets using common fluorophores in the Green, Yellow, Orange, Red, Crimson and Far Red channels. Applying the CXTM can improve cluster separation and support more reliable threshold placement and quantification.

Well-separated 1D scatterplots in amplitude-based multiplexing depend on minimizing optical crosstalk between channels. Before applying custom crosstalk compensation, crosstalk can be reduced by controlling the brightness of positive signals (see Table 1 in step 2). This is particularly important for the Yellow, Orange and Red channels, where optical crosstalk can substantially affect signal separation. Keeping signal intensities in these channels lower than in the other channels can improve cluster resolution.

Important note: To create a custom crosstalk matrix, the QIAcuity software requires both the singleplex reference reactions and the assay-free reaction-mix. The reference reactions must be defined as singleplex reactions in the QIAcuity Software Suite, even if they utilize amplitude-based multiplexing. If any reference well is defined as an amplitude multiplex or if the assay-free reaction-mix wells are missing, the "Create CXTM" option will not be available.

Generate the CXTM using the following workflow:

1. **Prepare the reference reactions**

- Prepare a separate reference reaction for each fluorescent dye in both the amplitude-multiplexed and non-amplitude-multiplexed channels. If amplitude multiplex assays will be used in a channel, combine both assays in that channel into a single reaction at the assay concentrations intended for the final multiplex. This allows the software to capture the individual fluorescence profiles generated by each color in the final multiplex reaction.
- Include each target at 125–1250 copies/µL to produce ~25–75% positive partitions per well and to generate clear positive populations for both assays, as shown in Figure 1.
- Include a reference reaction containing the reaction mix without any assays. This no-assay control accounts for fluorescence contribution from the passive reference dye of the mastermix.
- Use the same assay concentrations, reaction chemistry, cycling conditions and imaging settings intended for the final multiplex assay.

2. **Create the CXTM in the QIAcuity Software Suite**

- Define the single-color reference reactions as singleplex reaction mixes in the Plate Configurator and assign them to the corresponding wells. Do not assign a reaction mix to the well containing the no-assay control mix.
- Create a new CXTM and select the reference reactions from the completed dPCR run.
- Check that each reference well contains the expected negative and positive populations and that the threshold is placed beneath all three positive clusters, as shown in Figure 4.
- Save the CXTM as a reaction mix template with a clear name that identifies the assay set, concentrations, imaging settings and date.

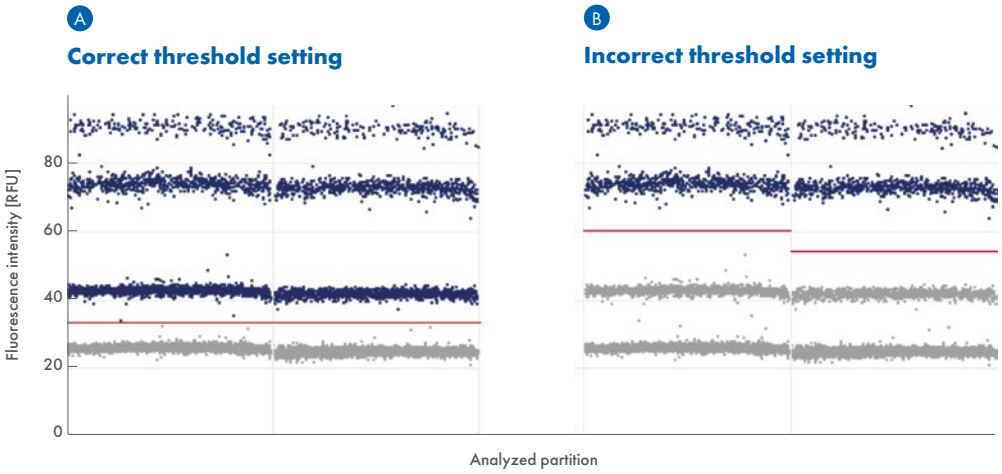
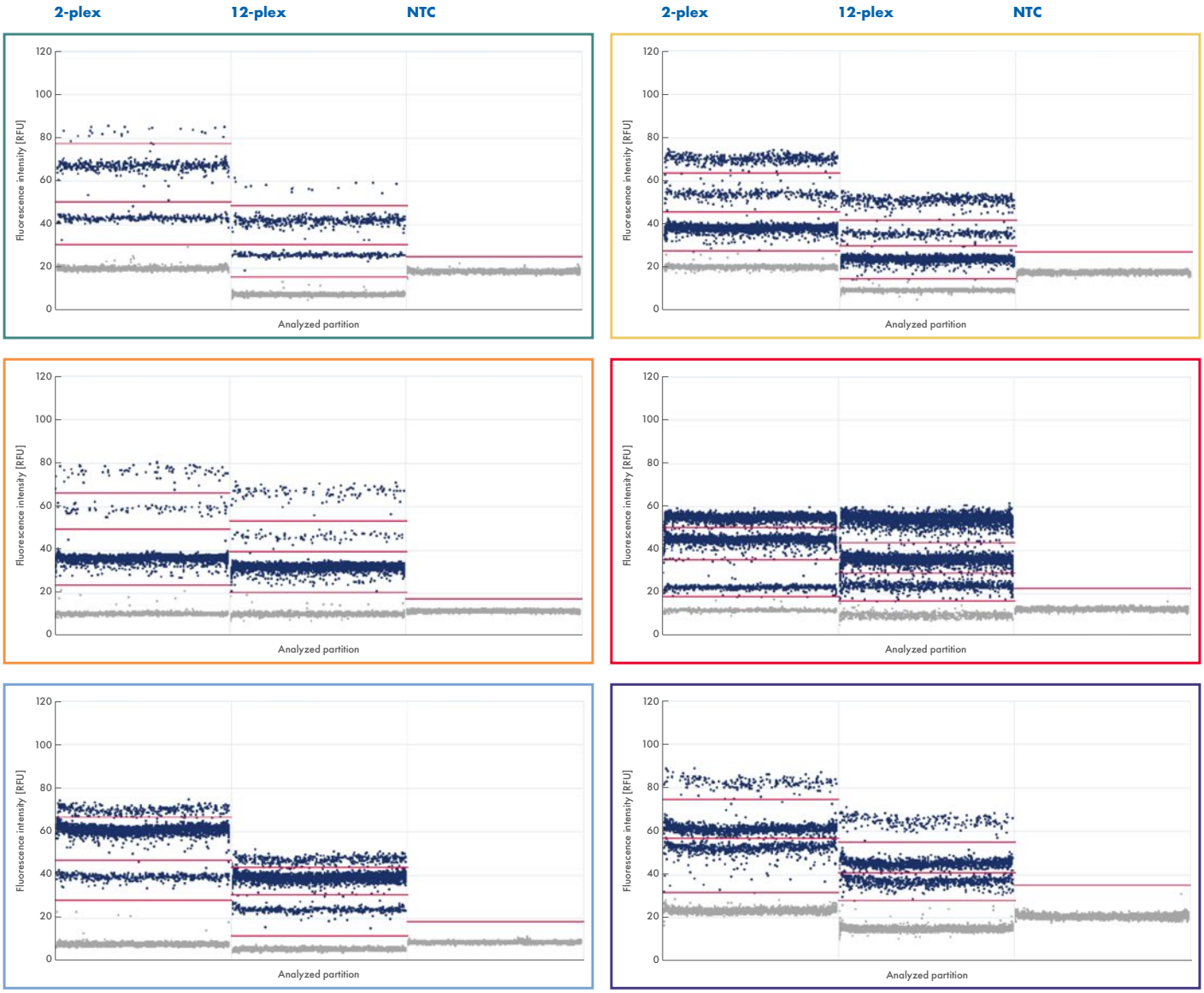


Figure 4. Threshold setting to generate custom crosstalk matrix. Thresholds are required to separate positive signal from negative signal, which informs the crosstalk compensation for each channel. In amplitude-based duplex reactions, thresholds must be positioned below all three clusters of positive signal (A) to achieve correct crosstalk compensation. Failure to set thresholds appropriately (B), as seen by the two negative clusters in gray, can lead to errors in crosstalk compensation.

3. Combine the optimized duplexes in the final 12-plex

- Combine the validated duplex assays to assemble the final multiplex assay with up to 12 targets. Include positive control material that contains all targets at concentrations between 50 and 6000 copies/µL, ideally between 125–1500 copies/µL. Also include no-template controls.
- Run the multiplex reaction together with single-color duplex reactions for all targets.
- Import the reaction mix containing the CXTM created in Step 2 in the "Reaction Mixes" tab by clicking on "Import from template". Apply the imported mix to the wells containing the assay mix and analyze the run by enabling Amplitude Multiplexing in the relevant wells and channels.
- Confirm that the three expected amplitude populations in each active channel are clearly separated.
- Confirm that target quantification remains consistent between multiplex and single-color reactions, as shown in Figure 5.
- Accept the final multiplex only if all colors show clear separation for the three expected positive populations, controls behave as expected and target quantification remains consistent with the corresponding singleplex reactions.

Important note: Generate a new CXTM whenever there are changes to the fluorophores, assay composition, assay or probe concentrations, reaction chemistry or imaging settings. A matrix is valid only for the experimental configuration used to generate it. For more information on setting up a custom crosstalk matrix, download our technical note.



	Measured copies/µL	
Target Gene	**2-plex**	**12-plex**
SLIT2	203.0	196.3
ZEB1	146.3	144.3
CDKN1B	359.6	365.3
HSPA1A	3059.4	3065.8
TBP	65.9	75.5
RPL13A	2571.9	2906.8
VIM	5043.4	5340.8
SLC2A1	2082.7	2156.7
RHOA	1989.9	2173.2
TFRC	328.7	363.6
CTNNB1	751.5	726.7
TGFB1	422.5	444.1

Figure 5. Transition from single-color amplitude multiplex to a 12-plex reaction. For the final higher-order multiplex setup, all six single-color duplexes were combined and analyzed using the custom crosstalk matrix. Quantification obtained in the individual duplex reactions was compared with that obtained in the final 12-plex reaction. The example shows a 12-plex assay performed using Universal Human Reference RNA and targeting 12 human genes: *SLIT2* and *ZEB1* in the Green channel; *CDKN1B* and *HSPA1A* in Yellow; *TBP* and *RPL13A* in Orange; *VIM* and *SLCA1* in Red; *RHOA* and *TFRC* in Crimson; and *CTNNB1* and *TGFB1* in Far Red. Border colors for each plot represent assigned fluorophore channels and correspond to the target name highlighted in quantification table.

Ordering information

Product	Contents	Cat. no.
QIAcuity One, Four or Eight Platform System	Digital PCR instrument for detecting up to 5 fluorescent dyes or up to 8 fluorescent dyes operating under software version 3.1 or higher, enabling up to 12-plex analysis	911021, 911042, 911052
dPCR RNA-Plex Assays	One tube with lyophilized assay, dye/fluorophore configurable, 250 to 750 reactions (12 µl reaction in Nanoplate 8.5K)	250336, 250337
QIAcuity OneStep High Multiplex PCR Kit	4x OneStep High Multiplex PCR Master Mix, 100x OneStep High Multiplex RT Mix, Enhancer GC, RNase-free water, QN Internal Control RNA; for 100 (or 500) reactions in Nanoplate 26K and 333 (or 1666) reactions in Nanoplate 8.5K	250135, 250136

Find out more about the QIAcuity Digital PCR System:
www.qiagen.com/qiacuity

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