Improving accuracy in multiplex dPCR with the custom cross talk matrix feature of QIAcuity® Software Suite 3.0 and 3.1

Introduction

Highly multiplexed dPCR is a powerful technique that amplifies and detects multiple targets within a single reaction. By combining multiple PCR assays, you can maximize sample use while simultaneously reducing time and reagent consumption and accelerating the process of obtaining high-quality data. One unwanted phenomenon can affect multiplex dPCR: cross talk. This is when the spectra of fluorescent dyes overlap into neighboring optical channels, distorting results and leading to inaccurate quantification.

The Custom Cross Talk Matrix (CXTM)

The amount of cross talk in a QIAcuity dPCR reaction depends on multiple factors, such as the concentration of the fluorescently labeled hydrolysis probes, the type of fluorescent dyes attached to the hydrolysis probes and the chosen imaging settings. When the recommended dyes and imaging settings are used in a QIAcuity dPCR reaction, the QIAcuity Software Suite automatically compensates for unwanted cross talk between neighboring channels (Green, Yellow, Orange, Red, Crimson and Far Red). However, if non-recommended dyes or imaging settings are used in any channel, the default QIAcuity cross talk compensation may not suffice and cross talk between neighboring QIAcuity channels may result in additional unexpected populations of signal.
These unwanted signals could lead to false positives that impact thresholding in 1D and 2D scatterplots.
The same applies to reactions that use Long Stokes-Shift (LSS) dyes. In these instances, use the enhanced and dynamic custom cross talk compensation feature of the QIAcuity Software Suite. With this new feature, you can create a custom cross talk matrix tailored to as many as eight fluorescent dyes, including two LSS dyes.

How the Custom Cross Talk Matrix works

Optical cross talk is corrected by considering each fluorophore individually. To let the image analysis algorithm capture the fluorescence profile of each dye, run all the assays as simplex (also known as singleplex) reactions. Remember, the passive reference dye also contributes to cross talk. To correct this, add wells only containing master mix (no fluorescently labeled assays) to the plate.

The custom cross talk compensation feature employs user-defined reaction mixes for its calculations. As a first step, to define a CXTM, add reaction mixes for all simplex reactions to the plate. To define mixes, open the “Plate configurator”, define mixes under “Reaction mixes” and add them to the plate via the “Plate layout” environment on the QIAcuity Software Suite. ▷

To access the CXTM configurator, use the “Analysis” menu; therein there will be an option named ”Create CXTM.” ”Create CXTM” is available (active) only for plates fulfilling the pre-requisites for custom cross talk matrix creation. This option is also available for plates in the “Plates Overview.”

Once in the ”Create CXTM”, the first step is to select the wells for the reference channel. These are the wells containing master mix only (Figure 1).

Next, establish the target channels. To do this, select the wells containing simplex reactions for the same target (for example, FAM assays in the Green Channel) (Figure 2).

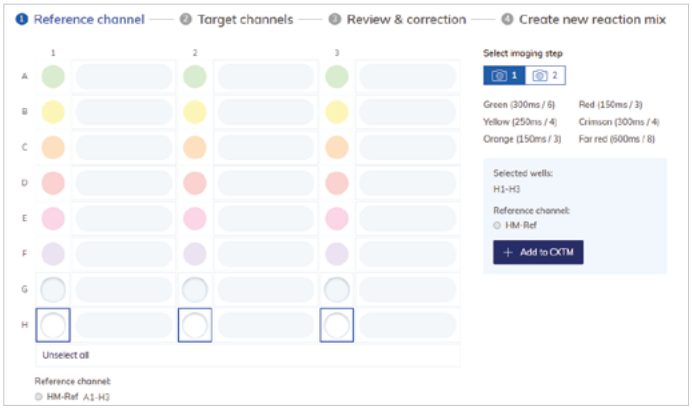

Figure 1. First step of generating a custom cross talk matrix. The well/s containing master mix are selected.

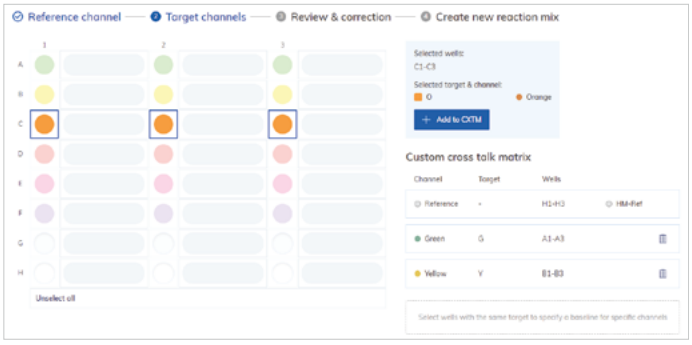

Figure 2. Second step of creating a custom cross talk matrix. All wells containing simplexes of the same target are selected and added to the CXTM.

The third step is “Review & Correction.” For this, review the 1D scatterplots for each channel. If necessary, adjust the threshold settings (Figure 3).

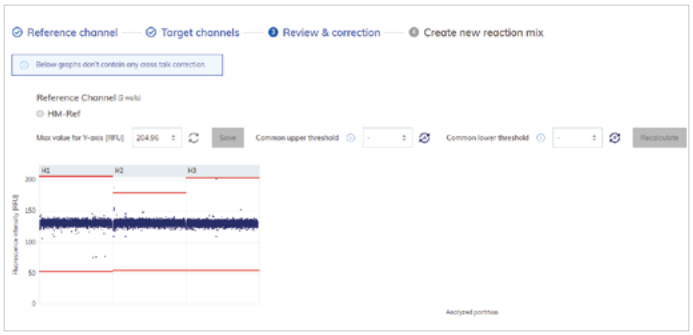

Figure 3. Third step of generating a custom cross talk matrix. The 1D scatterplots are displayed per target and you can adjust thresholds.

Finally, name the new reaction mix and template. Then, save it. This completes the custom cross talk matrix for a specific multiplex reaction (Figure 4).

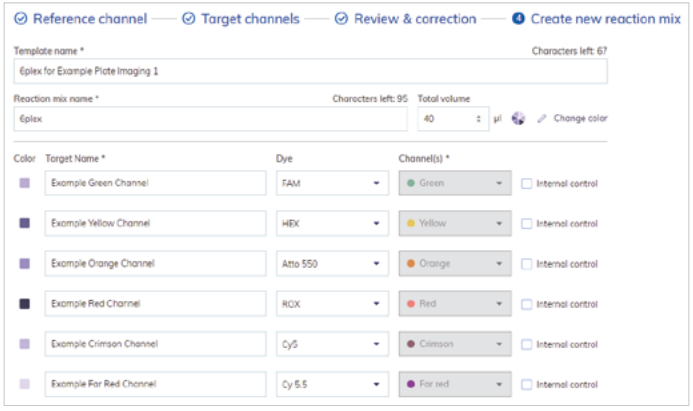

Figure 4. Last step of creating a custom cross talk matrix. A new multiplex reaction mix is created based on all simplexes. A template name and reaction mix name are defined.

To apply the reaction mix template to a new multiplex reaction, use the "Import from Template" function in the “Reaction mixes” tab. Select the desired reaction mix template and click “import.” The reaction mix including CXTM is added to the plate and can be assigned to the plate layout.

Once these changes are saved, you can continue to set up the experiment. In case of a finalized experiment, the Software Suite automatically recalculates the data. The cross-talk-compensated data are then displayed in the analysis menu. For further information, please refer to the *QIAcuity User Manual*.

Experimental considerations

Consider these factors when using the custom cross talk compensation feature. First, a CXTM is "reaction mix" specific and can only be re-used for the specific combination of assays from which it was created. If the multiplex conditions are altered (e.g., a new assay, a different dye or different concentrations of fluorescent probes are used), create a new CXTM.

The same is true for the imaging settings. If a plate is imaged with multiple settings or on different instruments, create a separate CXTM for each imaging.

As mentioned above, each assay in a multiplex must also be run in simplex reactions. For this, ensure that template concentrations for each target are about 25 to 75% positive partitions per well (~250 to 1500 cp/µL). Additionally, add wells containing master mix only. This enables the custom cross talk algorithm to estimate cross talk from and into the reference channel.

Cross talk estimation is highly dependent on the estimation of background fluorescence. For optimal background estimation, make the simplex reactions (used to train CXTM) as follows: Add all probes or all assays used in the multiplex. Then, build simplex reactions by adding amplification targets separately. However, this is only possible when individual targets are available.

Always use the correct matrix. If the wrong reaction mix, exhibiting the wrong CXTM has been assigned, re-assign the correct reaction mix and re-apply the custom cross talk compensation by re-analysis of the data.

When detecting seven or eight targets (utilizing the six standard channels plus one or two channel combinations for Long Stokes-Shift dyes), QIAcuity Software does not apply default cross talk compensation for the seventh and eighth channels (Figures 5 and 6). Create a CXTM for 7- or 8-plex reactions. If no custom cross talk matrix is employed, multiple populations of false positive signals appear in the 1D scatterplots for each channel. Applying a CXTM eliminates interfering signal, resulting in clean 1D scatterplots that show a single band of true positive signal (Figures 5 and 6).

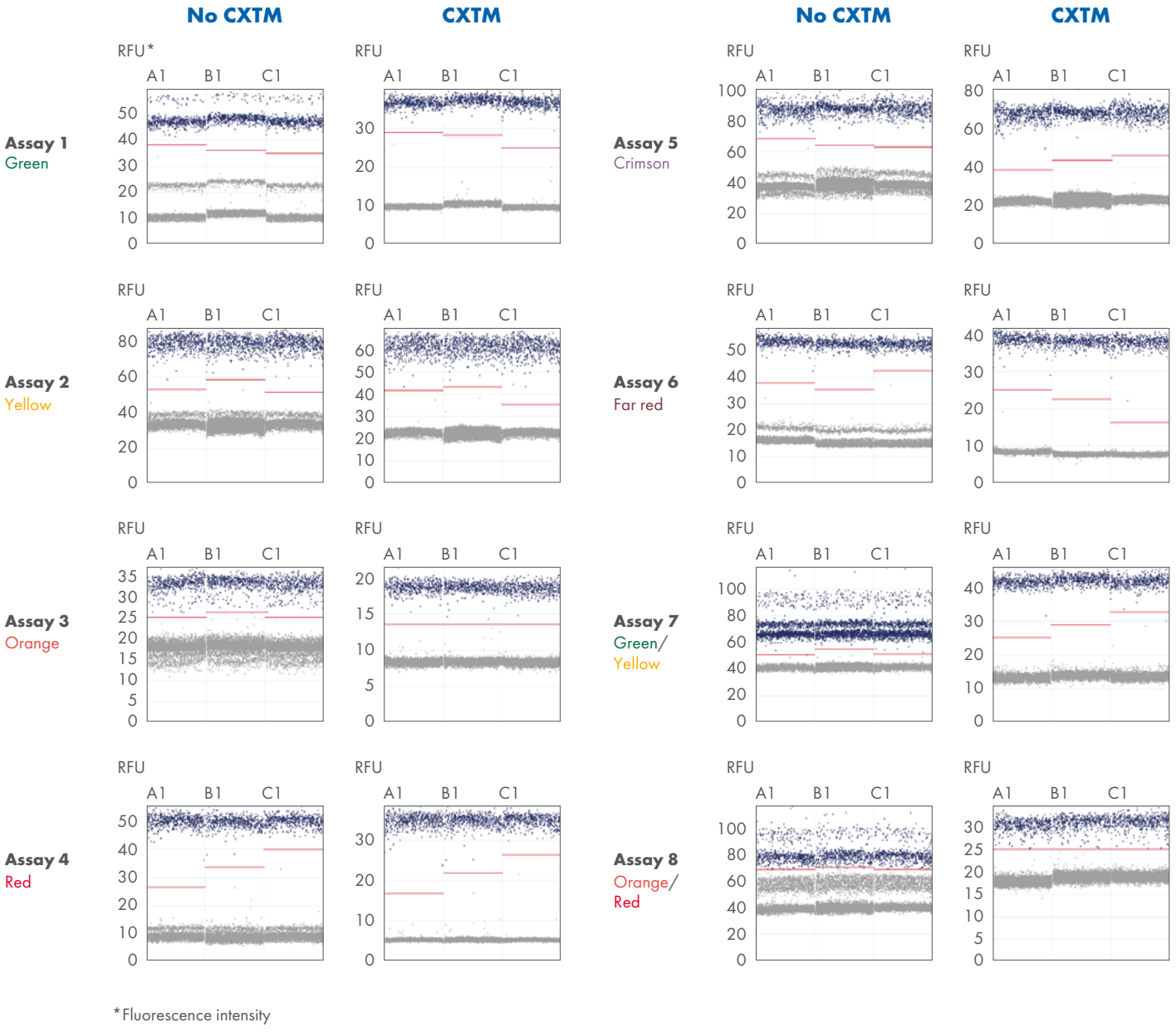

Figure 5. Custom cross talk compensation in QIAcuity Software 3.0 and 3.1 reduces false positives and improves the signal-to-noise ratio. 8-plex exemplary data with 8.5K 96-well Nanoplate using gDNA-based assays. In case of "No CXTM", the QIAcuity Software Suite has applied default cross talk compensation for the standard channels (assays 1–6). This default cross talk matrix is not applicable for the seventh and eighth channel.

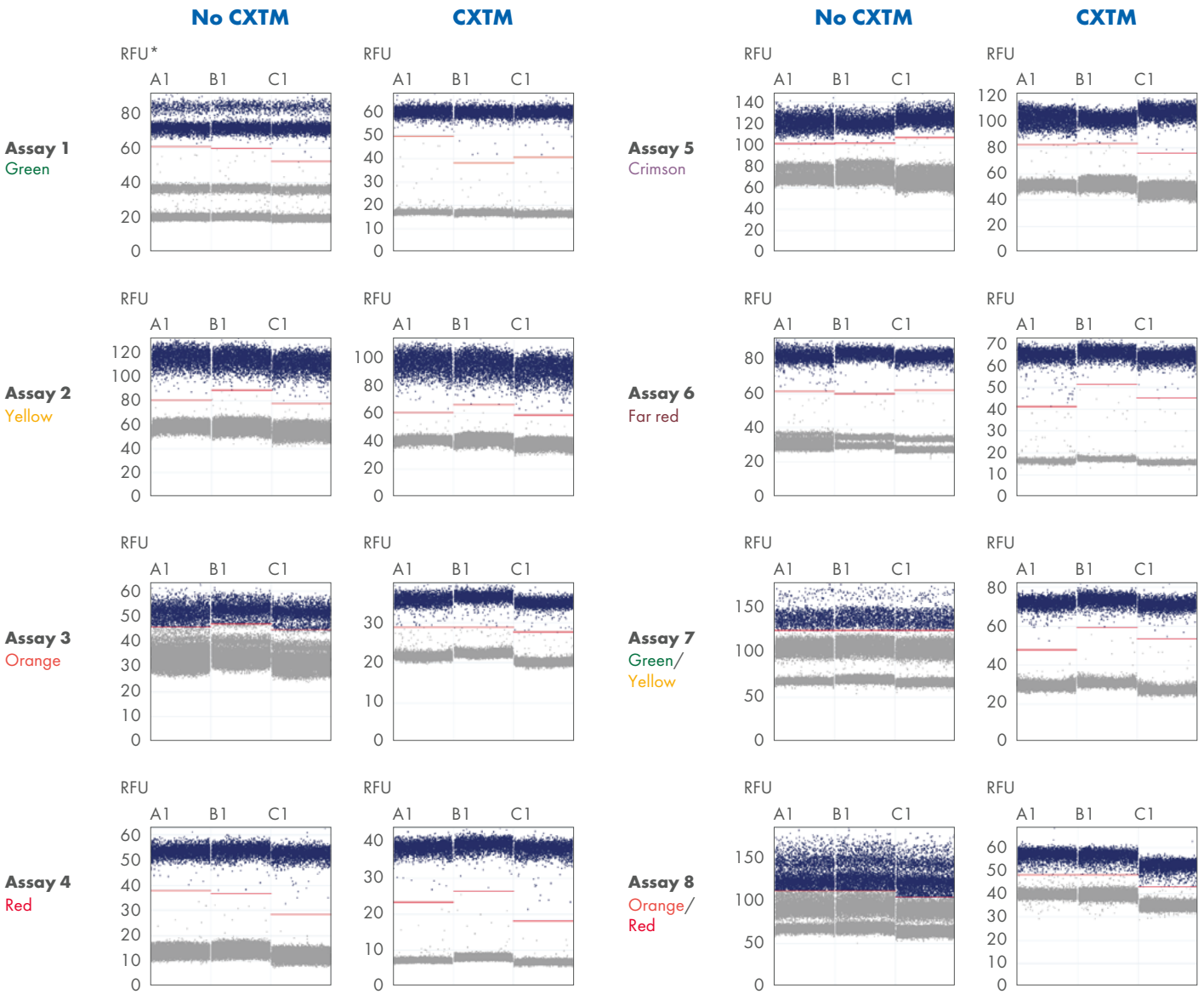

Figure 6. Custom cross talk compensation in QIAcuity Software 3.0 and 3.1 reduces false positives and improves the signal-to-noise ratio. 8-plex exemplary data with 26K 24-well Nanoplate using gDNA-based assays. In case of "No CXTM", the QIAcuity Software Suite has applied default cross talk compensation for the standard channels (assays 1–6). This default cross talk matrix is not applicable for the seventh and eighth channel.

Troubleshooting

High-multiplex dPCR is a powerful tool that may require optimization. If you're unsatisfied with your results after applying a CXTM, troubleshoot using the following advice.

Please ensure that the assays used to train the custom cross talk matrix are the same as those used in the multiplex wells. Furthermore, check that the threshold is correctly placed between negative and positive bands when creating the CXTM. If required, adjust thresholds in "Review and Correction" during CXTM creation (Figure 3). Results used to train the custom cross talk matrix (i.e., simplexes) should have little to no rain (that is, little to no signal between positive and negative signal clusters) and should not have a signal saturation flag.

As previously mentioned, imaging settings can also impact cross talk estimation. Remaining rain or unexpected double bands in the 1D scatterplots may result from suboptimal imaging settings used to train the custom cross talk matrix. As a general guideline, use RFU levels for the positive partitions in simplex reactions between 40 and 80 RFU with differences between related channels (for example, Green/Yellow and Yellow) not exceeding ~ 30%.

If cross talk between two dyes or neighboring channels is particularly challenging and the issue persists after modifying imaging settings, reducing the probe concentration can reduce bleedthrough fluorescence. If necessary, increase the exposure time for the specific channel where you have reduced the probe concentration.

These guidelines are particularly suitable for hybrid channels. Long Stokes-Shift dyes contribute to and are impacted by multiple channel cross talk. If assays in hybrid channels yield poor results (for example, concentration issues, multiple bands or unexpected shapes in 1D scatterplots), please review the setup parameters of the corresponding "standard" channels. For instance, for problems with assay in the Orange/Red channel, check and adjust the conditions for the assays in the Orange or Red channels (Figure 7). Importantly, if you change the concentration of any assay in future runs, create a new CXTM.

Finally, you can only add the CXTM configurator if the plate has already been processed and contains data.

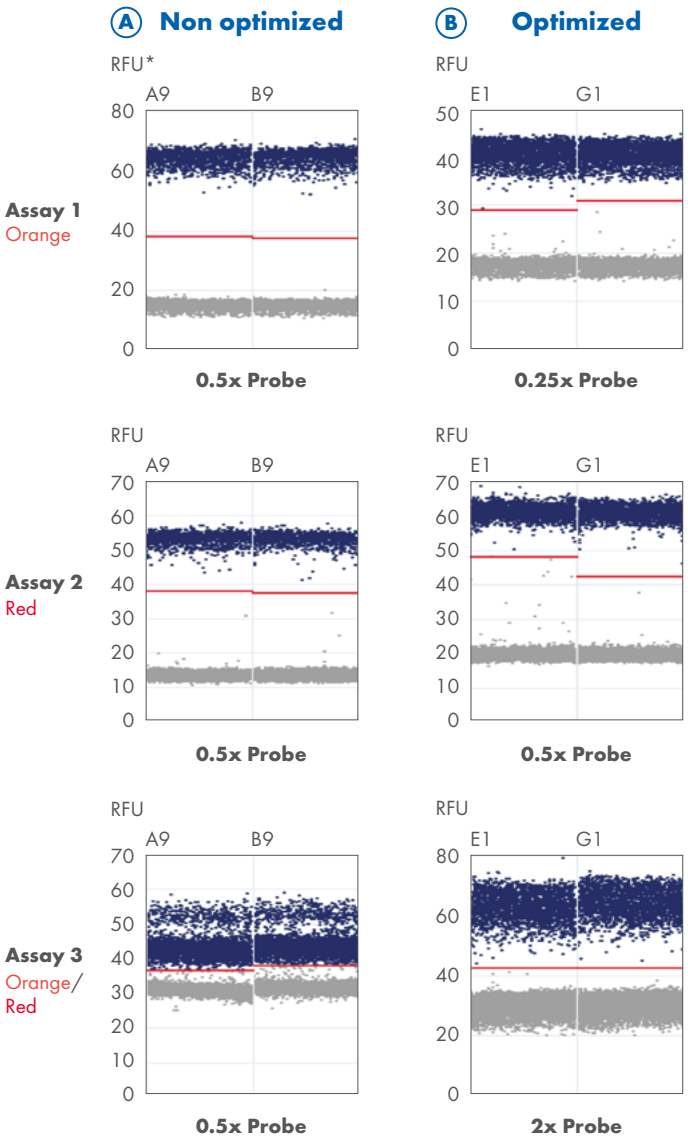

Figure 7. Exemplary 1D scatterplot data of multiplex optimization between Orange, Red and Orange/Red (8-plex experiment, but only relevant channels are displayed). 8-plex reaction mix using gDNA-based assays. **A** Orange, Red and Orange/Red assay probe of 0.5x concentration. **B** Orange, Red and Orange/Red assay, respectively, with 0.25, 0.5 and 2x probe concentrations.

Summary

Starting with QIAcuity Software Suite 3.0, the addition of new detection channels allows high-order multiplexing applications which are supported by the custom cross talk compensation feature of the software. Conveniently, saved CXTMs within reaction mixes can be easily applied to future datasets with the same setup.

By compensating for the overlap of fluorescent dye into neighboring channels, the new custom cross talk compensation feature means flexibility in dye choice and more confidence in quantifying data.

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

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