QIAcuity® Temperature Gradient Cycler to determine the optimal primer annealing temperature of a digital PCR assay

Introduction

In a PCR reaction, the annealing temperature is an important variable that defines the efficiency and specificity of the primer binding dynamics. Setting the annealing temperature too high usually leads to a more stringent primer binding, but lower PCR efficiency as primer binding events become less frequent. Setting the annealing temperature too low usually leads to more primer binding events and a more efficient PCR reaction, but also lower specificity as more unwanted primer binding events happen. Both cases can lead to a bias in absolute quantification events. The optimal annealing temperature is often set at a point where a sufficient balance between the two parameters (efficiency and specificity) is reached.

A new temperature gradient functionality

QIAcuity Software Suite version 2.5 newly enables an essential temperature gradient functionality. Gradient temperature functionality allows use of different annealing temperatures across the column of a QIAcuity 96-well 8.5k Nanoplate. This enables testing of different annealing temperatures within the same experiment to quickly and easily determine the ideal primer annealing temperature.

The temperature gradient functionality has been developed based on the integrated cycler module included in all QIAcuity instrument types. Users can immediately take advantage of this new feature after an upgrade to version 2.5 of the QIAcuity software. Once the optimal annealing temperature has been found, it can easily be transferred to all QIAcuity plate types.

Two predefined temperature ranges are provided within QIAcuity Software version 2.5. These ranges are optimized to address both Reverse Transcription (RT) and standard PCR-related applications, making other temperature ranges obsolete. The available ranges are:

- 55 to 42°C for RT-related applications
- 52 to 64°C for standard PCR-related applications

The temperature distribution per well (based on the selected gradient) can be previewed and is provided in the plate layout within the QIAcuity Software Suite. Please note that only one gradient cycling profile can be applied to one plate. When using a QIAcuity Four or QIAcuity Eight instrument, each plate may have one of the two gradient cycling profiles, independent of the other plates.

Experimental considerations

When determining the optimal annealing temperature, certain criteria should be met:

1. The absolute quantification (in cp/µl reaction) should reflect the expected values. If the quantification is too low, the PCR reaction was insufficient at producing signal above background levels. When the quantification is much higher than expected, then the annealing temperature was too low, allowing secondary primer binding events that lead to false-positive signals.

Note: Please make sure that the threshold is properly set, as threshold settings can influence the quantification results.

2. The signal-to-noise ratio should be sufficient to enable either manual and/or automatic definition of the threshold. This means that the mean RFU value from the positive cluster divided by the mean RFU value from the negative cluster should be high enough to allow for a clear separation of the two clusters. Generally, the higher the ratio, the better.
3. Some partitions on the plate might undergo a less efficient PCR reaction with lower RFU values than other partitions. On the 1D scatter plot, these partitions are classified as "rain" and are located in between the cluster of positive and negative partitions. In extreme cases, positive partitions can display RFU levels of real negative partitions. The overall recommendation is to select PCR conditions for which the amount of partitions in between the cluster of positive and negative partitions is reduced to a minimum.
4. The selected optimum temperature should allow the highest signal-to-noise ratio, minimal-to-no rain and reflect the expected concentrations. Finding the optimum temperature is also important for minimizing primer mismatch, which could lead to cross-hybridization issues in multiplex reaction mixes.

Example of the temperature gradient functionality

Performing a gradient cycling plate run helps determine optimal PCR conditions. For example, a gradient cycling plate run where 60°C annealing temperature (well C4/C5) gives the best signal-to-noise (S/N) ratio: the positive cluster is in mean at 60 RFU and negative cluster at about 10 RFU, so the S/N ratio is 6 (Figure 1 and Equation1).

$$\frac{\textbf{mean RFU positive cluster}}{\textbf{mean RFU negative cluster}} = \frac{60}{10} = 6$$

Equation 1. Calculating the S/N ratio for a gradient cycling plate run with 60°C annealing temperature.

Temperatures lower than 60°C show similar signal/noise ratios and amounts of rain but are less preferred due to the risk of cross-hybridization in multiplexing applications.

The selected optimal conditions can be verified with a new plate run using the standard cycling program with the optimum temperature found by using the gradient cycling functionality (Figure 2).

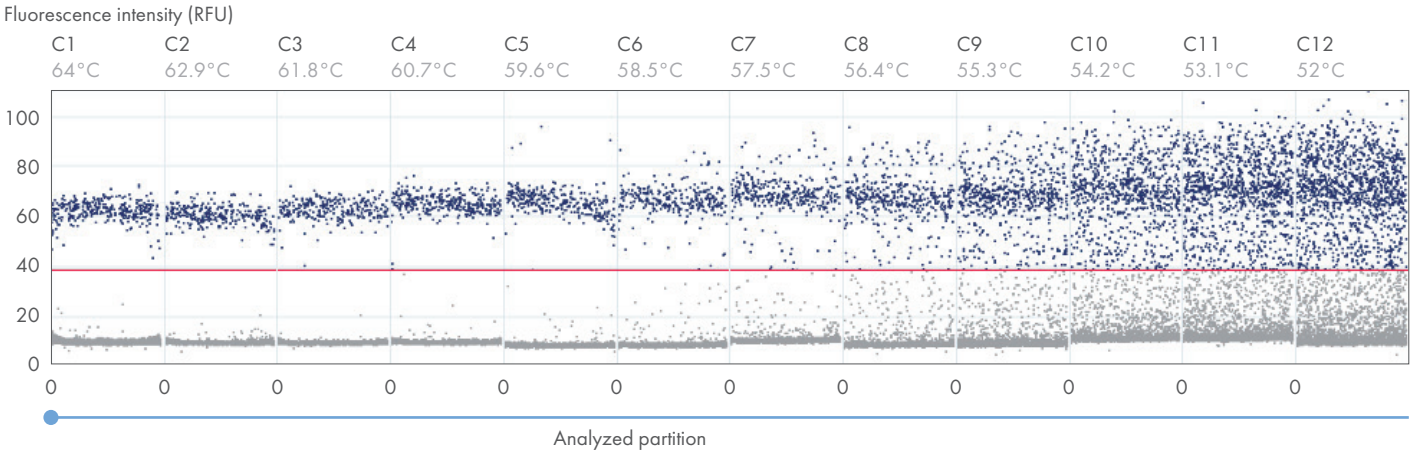

Figure 1. QIAcuity Software Suite version 2.5 example of 1D scatterplot results showing different S/N ratios and rain effect depending on the temperature gradient in the annealing step. The first and the last row (rows A & H) of a QIAcuity Nanoplate 8.5k 96-Well are not available for the gradient functionality and are disabled in the QIAcuity Software Suite plate overview when the temperature gradient functionality was selected.

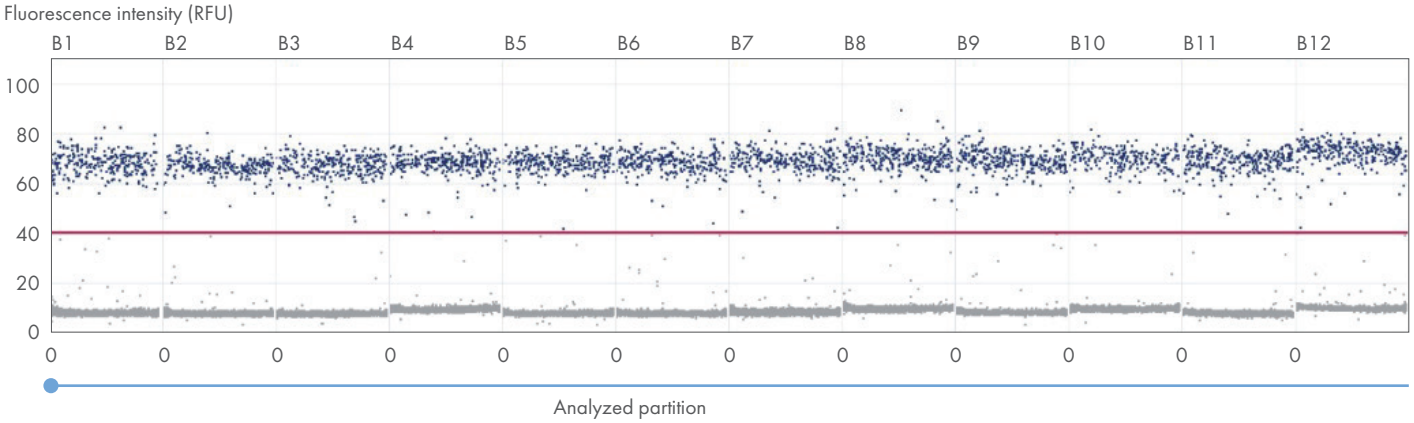

Figure 2. QIAcuity Software Suite version 2.5 example of 1D scatterplot with selected optimum temperature at 60°C based on gradient cycling results.

Summary/Conclusion

The QIAcuity Software Suite 2.5 offers a new, essential temperature gradient functionality for the integrated cycler of QIAcuity instruments. This functionality supports assay development by offering an easy way to determine the optimal primer annealing temperature. Two pre-defined temperature gradients are available, which have been optimized for both RT-PCR applications and standard PCR applications. After optimization of the annealing temperature using a QIAcuity Nanoplate 8.5k 96-well, the optimal temperature value can be transferred to any other Nanoplates available for the QIAcuity Digital PCR System.

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