Droplet Digital™ PCR: Guidelines for Multiplexing Using Bio-Rad's QX100™ Droplet Digital PCR System

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Digital PCR

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Abstract

Droplet Digital PCR (ddPCR™) enables accurate, precise, and sensitive quantification of specific nucleic acid sequences. In addition to the standard detection of two targets using two different fluorophores, it is possible to increase the number of targets detected by varying parameters that affect PCR efficiency and end-point fluorescence. In this case, we describe a method to multiplex assays by varying the concentrations of primers and probes or the type of fluorophores used. This allows users to expand the number of simultaneously detected targets up to four. Increasing the number of potential targets per test is a significant improvement for ddPCR, dramatically augmenting the information output of each sample.

Results

By diluting the assay used in ddPCR, it is possible to modify the amplitude of the positive droplets while retaining the ability to quantify the target (Figure 1). When using more than one assay per color, one of the assays is used at its normal dilution (1x) while the other is used at 60% of its normal dilution (0.6x, Figures 2A and 2B). When used in combination with a FAM assay this results in eight different clusters (eight possible combinations with three assays, Figure 2A). In order to confirm the identity of each cluster, the DNA was predigested by HaeIII, which cuts inside the primer sequences for the chromosome 10q1 (Chr10q1) assay. After ddPCR, four of the clusters disappear, confirming the predicted location for the different clusters.

A similar approach is used to detect four targets (tetraplex assay, Figures 3A and 3B). This results in 16 different clusters ($2^4 = 16$) where droplets can contain anywhere from zero to four different targets (Figure 3A). Upon dilution of the target DNA, the clusters containing four and three targets are dramatically reduced (Figure 3B). All assays retain their quantification capacity when used in multiplexed reactions.

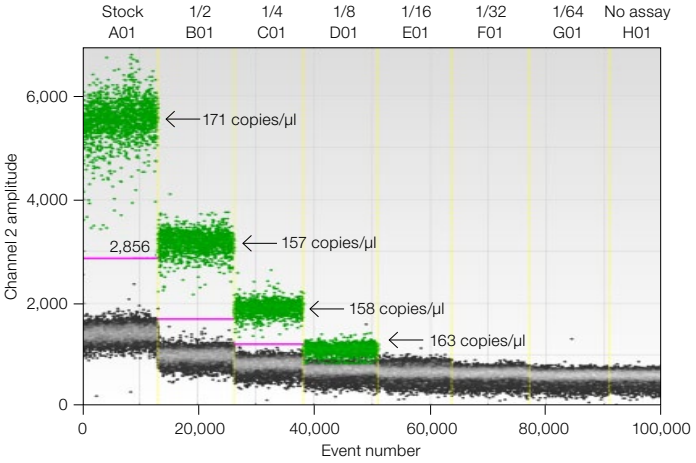

Fig. 1. Effect of assay dilution on fluorescence amplitude. Diluting the assay enables modification of the fluorescence intensity of the positive droplets while retaining the ability to quantify the targets with great reliability.

An alternative to varying assay concentrations is to use alternate fluorophores. Figures 4A–D outline the results obtained by combining assays labeled with Alexa Fluor 488, ATTO 488, TET, and HEX. Since the instrument is calibrated for FAM and HEX/VIC, alternate fluorophores can also be detected, but they will not align in a perfectly orthogonal matrix. This allows one to separate the targets and find the expected 16 different clusters. Single positive clusters are outlined in Figure 4A, double positives in Figure 4B, triple positives in Figure 4C, and quadruple positives in Figure 4D.

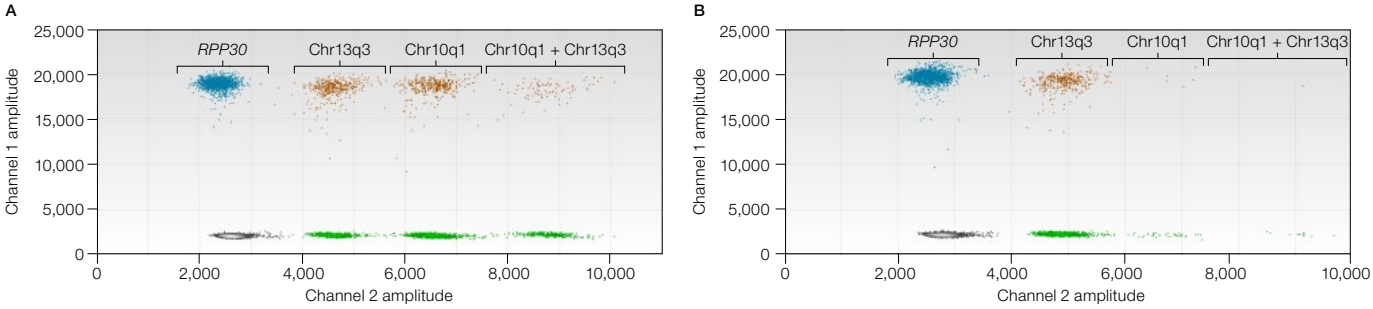

Fig. 2. Triplex assay using 1x *RPP30* (FAM) + 1x Chr10q1 (VIC) + 0.6x Chr13q3 (VIC) on a QX100 system. A, DNA was digested by AluI (cuts outside amplicons). Eight different populations were detected: one negative, three single positives, three double positives, and one triple positive. The quantification of all three targets is equivalent (euploid genome). Chr13q3 is detected at a lower level of fluorescence due to its lower assay concentration (0.6x instead of 1x). **B**, DNA was digested by HaeIII (cuts in between Chr10q1 primers). There is significant reduction of populations containing Chr10q1 positive droplets, therefore validating the detection pattern. This experiment also demonstrates that the QX100 system allows an additive effect of the fluorescence from two different reactions using the same fluorophore. Chr, chromosome.

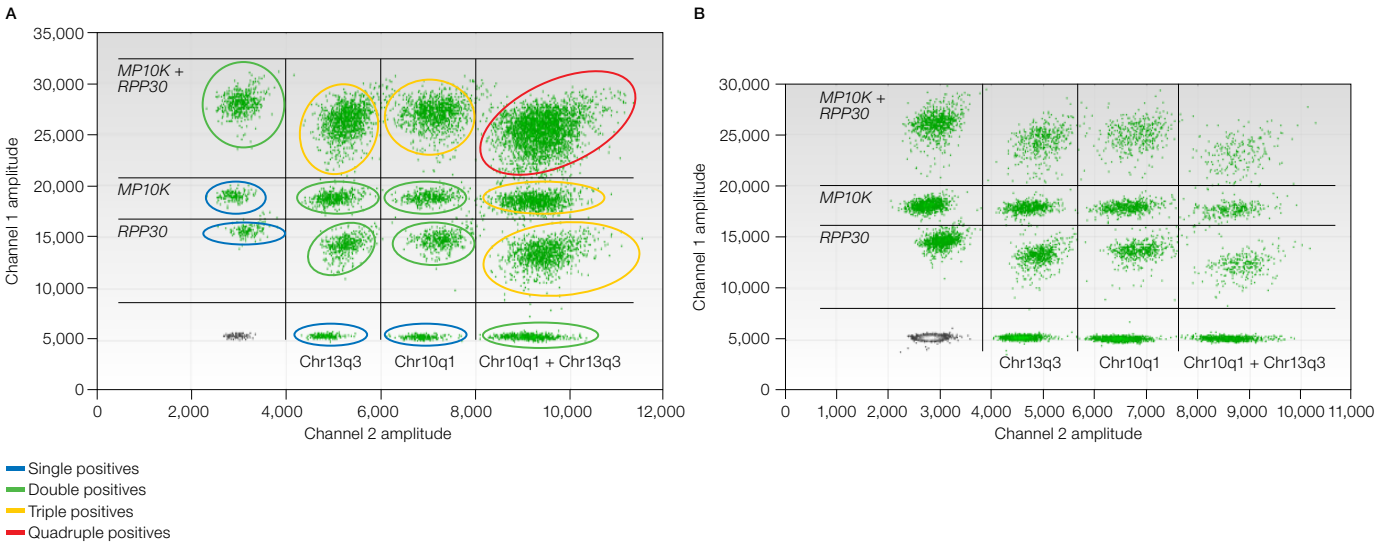

Fig. 3. Simultaneous detection of four different targets by ddPCR. A, tetraplex assay using 1.5x *MP10K* (FAM) + 1x *RPP30* (FAM) + 1x Chr13q3 (VIC) + 0.6x Chr10q1 (VIC) on a QX100 system. Sixteen different clusters are shown in this 2-D plot. The clusters represent different combinations of the detected targets (one negative cluster, four single positives, six double positives, four triple positives, and one quadruple positive). This illustrates the capacity of the QX100 system to separate discrete populations by levels of fluorescence using only two fluorophores. **B**, tetraplex assay, with template DNA diluted one-quarter, performed on a QX100 system. When loading one-quarter of the DNA amount in the reaction, we observed a reduction of positive populations, in particular the triple and quadruple positives. This illustrates that the QX100 system retains its ability to quantify targets in a multiplex setting. The capability to multiplex up to four targets should enable researchers to increase the scope of their research (more answers per well), as well as save time and reagents. Chr, chromosome.

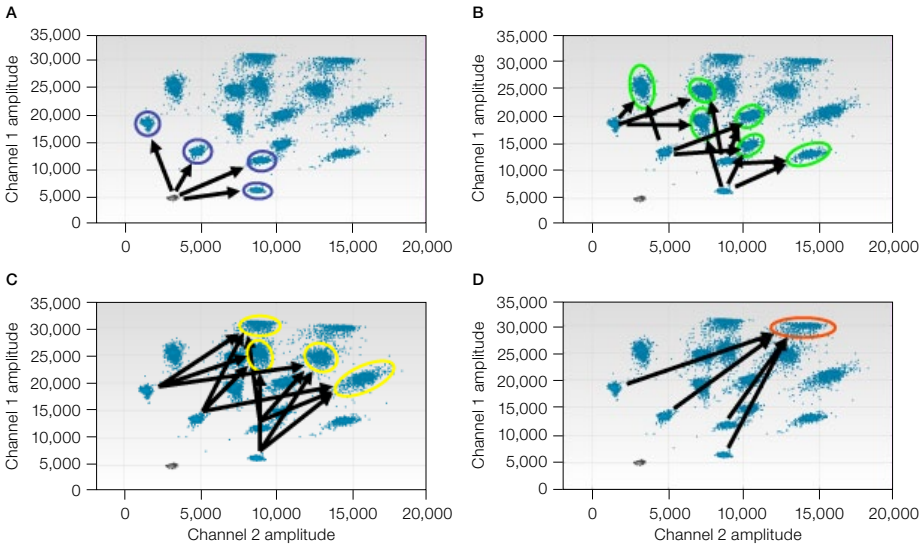

Fig. 4. Multiplex assay using fluorophore combinations to identify different populations on a QX100 system. These charts outline the relationship between different populations: single (**A**), double (**B**), triple (**C**), and quadruple (**D**) positives. This labeling was confirmed by performing triplex ddPCR reactions, where one of the different assays was omitted from the reaction (data not shown).

Conclusions

- 2 different multiplex assays using the QX100 system were established
- Multiplexing by assay concentration allows for orthogonal distribution of clusters, but requires optimization of fluorescence amplitudes
- Multiplexing with nontraditional fluorophores can be performed without changing assay concentrations, but the 16 clusters are distributed in a nonorthogonal matrix
- Expanding the number of assays opens up new possibilities for ddPCR technology applications

For more information, visit
www.bio-rad.com/web/ddPCRmultiplexing.

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