# Evaluating different template types and their distribution in QIAcuity™ Nanoplates for more accurate quantification by digital PCR

Abstract: The success of digital PCR (dPCR) relies on the random partitioning of the nucleic acid sample into individual reactions. For the vast majority of QIAcuity dPCR applications, template DNA is uniformly distributed throughout the microfluidic QIAcuity Nanoplate reaction chambers. However, structurally complex DNA molecules >30 kb remain unevenly partitioned, leading to the over-estimation of template concentration. By adding restriction enzymes directly to the reaction mix, large templates can be fragmented into smaller sizes, enabling even template distribution and more accurate quantification. When adding restriction enzymes to the reaction mix, users must ensure they do not cut within the amplicon sequence.

Introduction

The QIAcuity Digital PCR System seamlessly integrates a standard, multi-step dPCR workflow (partitioning, thermocycling and imaging) into a walk-away automated platform with minimal hands-on time. It can be used for various applications, including rare mutation detection, gene expression studies and copy number variation (CNV) analysis. Therefore, the system is designed to accommodate diverse sample types, such as PCR products, DNA extracted from formalin-fixed paraffin-embedded tissue (FFPE gDNA), cDNA and high-molecular-weight genomic DNA.

At the core of the QIAcuity workflow lies the proprietary microfluidic Nanoplate. Depending on the configuration, each Nanoplate well comprises 8,500 (8.5K) PCR reaction chambers (i.e., partitions) in a single well or 26,000 (26K) PCR reaction chambers evenly split across four sub-wells (Figure 1A). The DNA template is distributed throughout the Nanoplate wells in a priming step where the DNA-containing reaction mix is pushed through an input well such that it flows unidirectionally across rows of interconnected solid-state partitions (Figures 1B–C). A prerequisite for accurate dPCR quantification is the even, unbiased partitioning of the DNA template into individual reaction chambers. In this white paper, we evaluate the distribution of various sample types in Nanoplate wells with 8.5K and 26K partitions.
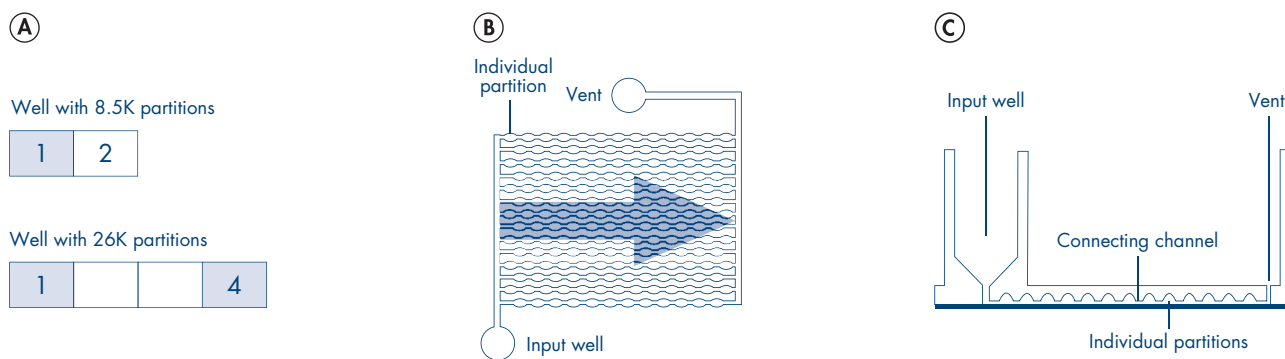
Figure 1. Schematic of a QIAcuity Nanoplate dPCR well. QIAcuity Nanoplate wells contain 8,500 (8.5K) or 26,000 (26K) partitions (**A**). In this study, the evenness of template partitioning was evaluated by comparing the abundance of PCR signal between the first and second half of 8.5K Nanoplate wells (depicted as 1 and 2) or the first and fourth sub-wells of 26K Nanoplate wells (depicted as 1 and 4). A hairline schematic depicts the key features of a generic Nanoplate well viewed from below (**B**) or in cross-section (**C**). During the priming step of the QIAcuity workflow, the reaction mix enters a Nanoplate well from an input reservoir. It moves unidirectionally (**B**, blue arrow) across rows of individual partitions, interconnected by channels. The reaction mix progresses to the opposite side of the dPCR well, where it ultimately reaches a vent channel.
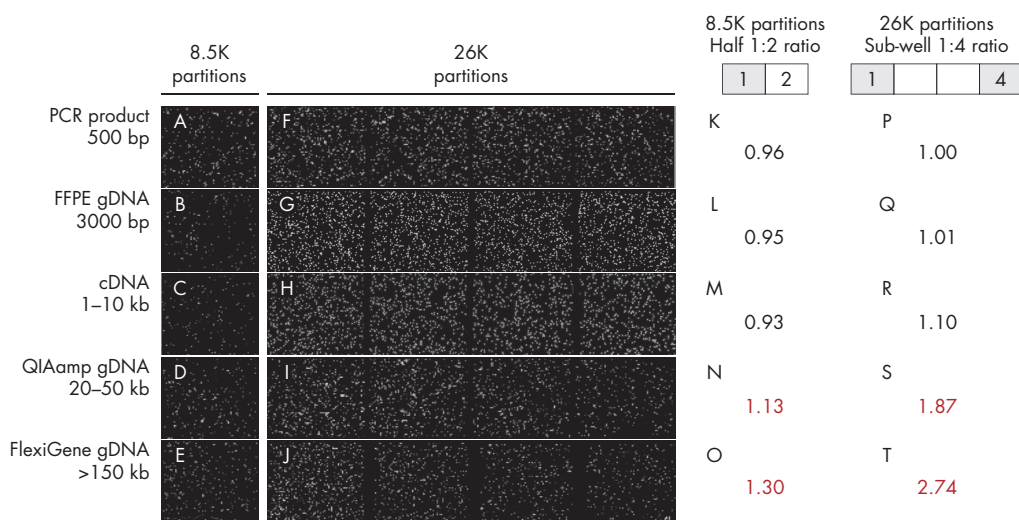
Methods

Enzymatic restriction of template DNA

Samples were prepared at the desired concentration before setting up the reaction mix. For the initial investigation into the distribution of various DNA templates in QIAcuity Nanoplates, depicted in Figure 2, the following assays and master mixes were used: PCR product, FAM QNIC, QIAcuity Probe; FFPE gDNA, HEX PIK3CA H1047R, QIAcuity Probe; cDNA, PFKL, QIAcuity EvaGreen®; QIAamp® gDNA, Texas Red™ ERBB2, QIAcuity Probe; FlexiGene® gDNA, Texas Red ERBB2. For experiments evaluating the fragmentation of FlexiGene DNA with restriction enzymes, the following assays and master mixes were used: FAM CTNNB1, FAM MYC, FAM VEGFA, Texas Red ERBB2, QIAcuity Probe; CTNNB1, MYC, VEGFA, QIAcuity EvaGreen. Restriction enzymes were added directly to the reaction mix in the presence of template DNA as well as a dye- or probe-based assay. Reactions were assembled at room temperature and incubated at room temperature for 10 minutes if restriction enzymes were added. dPCR was performed in QIAcuity Nanoplates.

Results and Discussion

For the majority of QIAcuity applications, the template was evenly distributed among the Nanoplate partitions. In QIAcuity reactions using PCR products, FFPE DNA, or cDNA as a template, a uniform distribution of PCR signal was observed in 8.5K (Figures 2A–C) and 26K (Figures 2F–H) Nanoplate reaction wells. For these reactions, the ratio of PCR signal between the first and second half of 8.5K wells (Figures 2K–M) and the first and fourth sub-wells of 26K wells (Figures 2P–R) was close to 1, indicating balanced template distribution. In contrast, images (Figures 2D, E, I, J) and distribution ratios (Figures 2M–O, Figures 2R–T) of PCR signal from reactions using intact human gDNA indicated that large DNA templates are unevenly partitioned in Nanoplate wells. More specifically, large DNA templates were spread out over a gradient, accumulating towards the first half and first sub-well of 8.5K and 26K Nanoplate wells, respectively. The extent to which large DNAs showed biased distribution to one side of a Nanoplate well is size-dependent. QIAamp gDNA, with an average size of 20–50 kb, had distribution ratios of 1.13 and 1.81 in 8.5K and 26K Nanoplate wells, respectively. FlexiGene gDNA, with an average size of >150 kb, accumulated to a much greater extent, having distribution ratios of 1.3 and 2.47 in 8.5K and 26K Nanoplate wells.

	8.5K partitions	26K partitions		8.5K partitions Half 1:2 ratio	26K partitions Sub-well 1:4 ratio
PCR product 500 bp	A	F		K 0.96	P 1.00
FFPE gDNA 3000 bp	B	G		L 0.95	Q 1.01
cDNA 1–10 kb	C	H		M 0.93	R 1.10
QIAamp gDNA 20–50 kb	D	I		N 1.13	S 1.87
FlexiGene gDNA >150 kb	E	J		O 1.30	T 2.74

Figure 2. The uniformity of DNA distribution in QIAcuity Nanoplates is size-dependent. Typical QIAcuity samples ranging from 500 bp to >150 kb in size served as templates in 8.5K and 26K QIAcuity dPCR reactions. Images of PCR signal from 8.5K (A–C) and 26K (F–H) Nanoplates indicate that smaller DNA templates are evenly distributed. In comparison, larger templates like intact human gDNA accumulate across a left-to-right gradient (D, E, I, J). For smaller DNA templates, the ratios of positive partitions between the first and second half (Half 1:Half 2) of 8.5K Nanoplate wells (K–M) and the first and fourth sub-wells (Sub-well 1:Sub-well 4) of 26K Nanoplate wells (P–R) are close to 1, indicating even template partitioning. For larger DNAs, Half 1:Half 2 and Sub-well 1:Sub-well 4 ratios exceed 1 and reveal that distribution bias is more severe for DNA >150 kb (O, T) than for DNA between 20–50 kb in size (N, S).

To investigate the impact of uneven template distribution on QIAcuity quantification, FlexiGene human gDNA was diluted to 100 copies/μl, based on UV absorbance. It was measured in 8.5K and 26K Nanoplates with three EvaGreen and three probe-based assays. In all 12 dPCR setups, the imbalanced distribution of FlexiGene DNA (Figures 3A, B) correlated with an over quantification of DNA concentration by 16 to 44% (Figures 3C, D). However, fragmenting the FlexiGene human gDNA before partitioning, by adding restriction enzymes directly to the reaction mix, resulted in even template distribution and accurate quantification in all 12 assay setups. (Figures 3A–D). Importantly, the addition of restriction enzyme to QIAcuity reaction mixes did not impact the distribution (Figures 3E, G) or quantification (Figures 3F, H) of a much smaller 500 bp template in 8.5K or 26K Nanoplates.
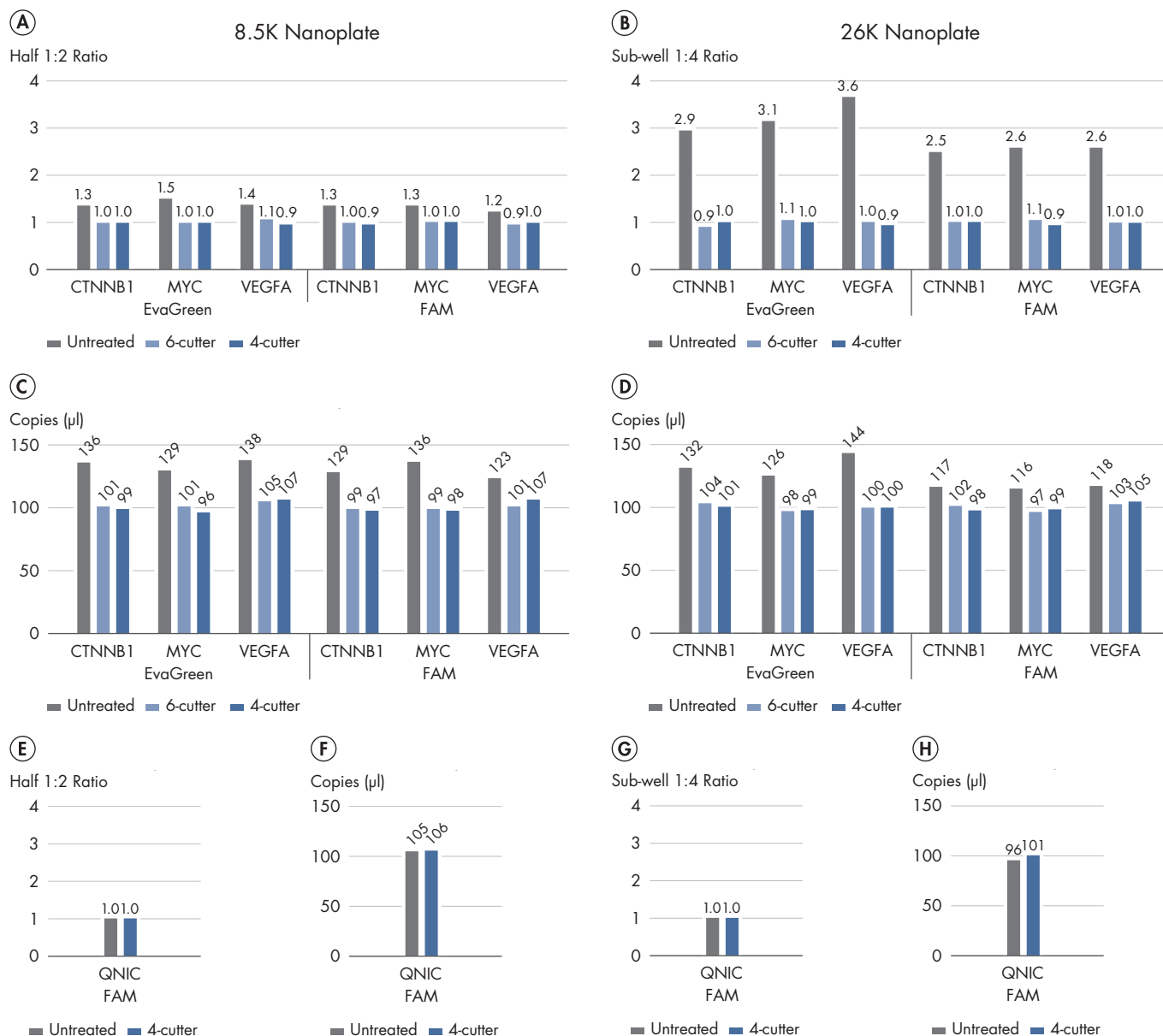
Figure 3. Uneven distribution of large DNA in QIAcuity Nanoplates leads to inaccurate template quantification. FlexiGene human gDNA, with an average size >150 kb, was diluted to a final concentration of 100 copies/μl in QIAcuity reaction mixes. Independent dPCR reactions targeting three single-copy genes (CTNNB1, MYC, VEGFA) were performed using intercalating dye (EvaGreen) or probe-based (FAM) assays in 8.5K and 26K QIAcuity Nanoplates. Untreated FlexiGene template accumulated in the first half (Half 1:Half 2 Ratio) of 8.5K Nanoplate wells (A) and the first sub-well (Sub-well 1:Sub-well 4 Ratio) of 26K Nanoplate wells (B), leading to over quantification of DNA concentration (C, D). The addition of restriction enzymes directly to the dPCR reaction mix did not artificially skew DNA distribution or quantification results. Template distribution and accurate quantification in dPCR reactions verified using a 500 bp PCR amplicon (QNIC) as a template with and without digestion (E–H). For all setups, n≥8.

By recognizing specific 6-nucleotide (6-cutter) or 4-nucleotide (4-cutter) sequences, restriction enzymes added directly to the QIAcuity Probe (Figure 4A) or QIAcuity EvaGreen (Figure 4B) reaction mixes reduced the average size of FlexiGene template from >150 kb down to ~4096 and ~256 bp, respectively.
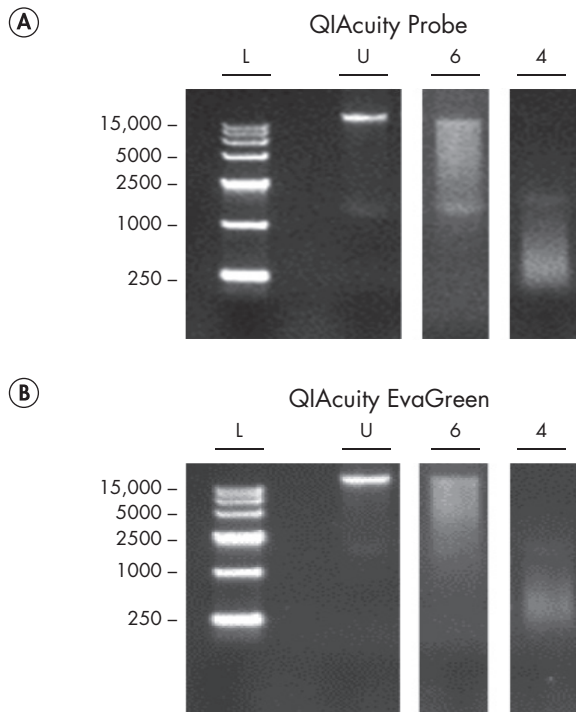
Figure 4. Restriction enzymes fragment DNA directly in QIAcuity Probe and QIAcuity EvaGreen mixes. The activity of two restriction enzymes was assayed in 12 µl QIAcuity Probe (**A**) or QIAcuity EvaGreen (**B**) reaction mixes containing 210 ng of FlexiGene gDNA. Untreated DNA (U) appeared as a high-molecular-weight band on a 0.8% agarose gel. Addition of EcoRI-HF®, a 6-cutter (6) restriction enzyme, or AluI, a 4-cutter (4) restriction enzyme, fragmented the FlexiGene DNA to average sizes of ~4096 and ~256 bp, respectively. Reactions were carried out at room temperature for 10 minutes. EcoRI-HF and AluI were added at concentrations of 0.25 and 0.025 U/µl, respectively. GelPilot HighRange Ladder (100) was used as a size ladder (L). Refer to the QIAcuity Applications Guide for additional information on restriction enzymes compatible with the QIAcuity dPCR mixes.

Having shown that fragmenting FlexiGene DNA to <5 kb results in even distribution and accurate quantification, we set out to identify the cut off size at which distribution and quantification issues begin. Various restriction enzymes were added directly to QIAcuity Probe mixes containing an ERBB2 assay and FlexiGene gDNA, diluted to 100 copies/µl, based on UV absorbance.

Restriction enzymes were chosen such that ERBB2-containing fragments between 0.3 and 45 kb were generated. dPCR reactions performed in 26K Nanoplates revealed that ERBB2-containing fragments between 0.3 and 31 kb are uniformly distributed (Figure 5A) and accurately quantified (Figure 5B). With a Sub-well 1:Sub-well 4 ratio of 1.6, the 45 kb ERBB2-containing fragment is unevenly partitioned, correlating to inaccurate quantification. However, the distribution bias of the 45 kb ERBB2 fragment is not as severe as that of intact FlexiGene gDNA.
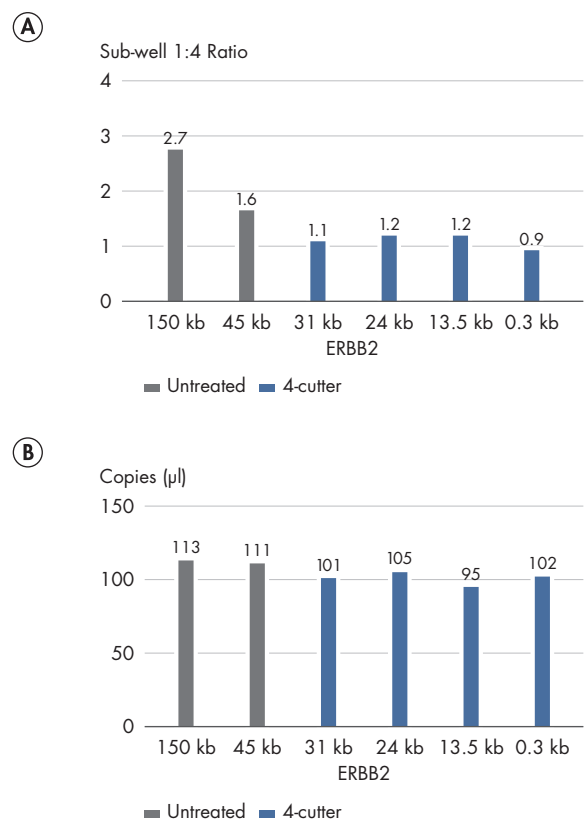
Figure 5. DNA fragments <30 kb are evenly distributed and accurately quantified in QIAcuity Nanoplates. FlexiGene human gDNA, with an average size >150 kb, was diluted to a final concentration of 100 copies/µl in QIAcuity Probe reaction mixes containing an ERBB2 assay. Restriction enzymes were added directly to the mixes, such that ERBB2-containing fragments between 0.3 and 45 kb were generated. dPCR was performed in 26K QIAcuity Nanoplates, revealing that ERBB2-containing fragments between 0.3 and 31 kb are uniformly distributed (**A**) and accurately quantified (**B**). In contrast, 45 kb ERBB2 fragments are unevenly partitioned, which correlates with inaccurate quantification. The uneven distribution of the 45 kb ERBB2 fragment is not as severe as that of intact FlexiGene gDNA. For all setups, n≥8.

Conclusion

In conclusion, our study provides a rationale for choosing restriction digestion as an optimal DNA fragmentation method for specific template types used for microfluidic nanoplate-partitioning in dPCR. Digestion using a restriction enzyme is useful when using high-molecular-weight templates with complex structures. Digestion can be carried out directly in the reaction mixes using high-fidelity restriction enzymes (4- or 6-nucleotide cutter enzymes) that do not cut within the amplicon region.

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