Application Note

Impact of template addition volume and analyzed volume on digital PCR sensitivity

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Introduction

In digital PCR (dPCR), a PCR reaction is split into a high number of individual reaction partitions (chambers or droplets), containing either 0, 1, or >1 template molecules per partition. After the partitioning, PCR amplification is performed. The subsequent fluorescent readout, enabled by intercalating dyes (e.g. EvaGreen®) or fluorescent hydrolysis probes (TaqMan™ probes), will classify each partition as negative (containing no target molecule) or positive (containing one or more target molecules). Subsequently, total absolute copies present in the reaction are calculated based on the number of negative and positive partitions using Poisson statistics. Digital PCR has been demonstrated to be superior to other PCR methods such as qPCR (quantitative PCR) for applications in which the highest level of sensitivity is required for the detection of ultra-low concentration templates where only a few copies of target may be present in a sample. There are several systems for digital PCR on the market, which differ in numerous aspects, including but not limited to 1) the number of detection channels, 2) the number of samples processed per run, 3) the types and number of partitions, 4) the template addition volume for a given reaction, and 5) the percentage of a given reaction that is analyzed. Among these variations in the digital PCR systems on the market, two factors are of particular importance in relation to the level of sensitivity that each system can achieve: 1) the total amount of template that can be added to each reaction for analysis (template addition volume) and 2) the amount of the reaction that is ultimately analyzed by the dPCR instrument.

The first and most important factor that affects sensitivity is the template addition volume simply because the more sample added to a reaction, the more template will be present for analysis. In the case of ultra-low template concentrations, the higher the template addition volume, the higher the probability that an ultra-low concentration template will be added to the dPCR reaction. The highest achievable template addition volume for a given technology is dependent on the concentration of the corresponding master mix for the given technology and the total reaction volume. Depending on the dPCR system, the template volume added to a reaction can differ vastly and can range from 6 to 26 µl. This variability is captured in Table 1, where the reaction volume and template addition volume of 3 dPCR systems on the market are presented.

The second factor that affects sensitivity is the percentage of the given sample that is analyzed in the given technology. This can vary across different systems and is referred to as 'dead volume' or 'non-analyzed volume'. 'dead-volume' is the volume fraction of a PCR reaction which is loaded onto a dPCR plate or chip, but is not, ultimately, transferred into the reaction compartments and therefore not subjected to subsequent analysis. The QIAcuity 26k nanoplate allows for the analysis of 21.6 µl which results in a 46% non-analyzed volume. The Applied Biosystems MAP16 plate configuration, on the other hand, allows for the analysis of 8.2 µl which results in a ~9% dead volume. The Bio-Rad QX200 likely has a dead volume of ~43 to 48%. This is calculated with the assumption of a 16,000 droplet count versus the ▶

theoretical droplet count maximum of 20,000 and using measured droplet volumes of 0.718 nl and 0.786 nl from two independent studies[1-2]. It recently has been speculated to which extent dead volume of a digital PCR system impacts the sensitivity of the dPCR reaction[3-4].

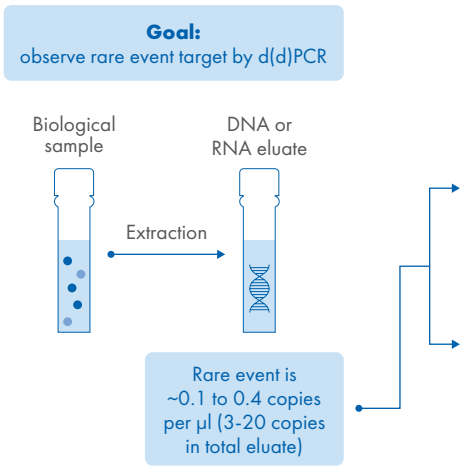
Table 1. Reaction volumes for three commercially available dPCR technologies

	QIAGEN Nanoplate 26k	MAP16 dPCR plate	ddPCR
Reaction volume	40 µl	9 µl	22 µl
Maximum Template Addition Volume (assuming 4x master mix and 10x assay)	26 µl	5.9 µl	14.3 µL
Reaction Volume Analyzed = Reaction Volume - non-analyzed volume	~21.6 µl	~8.2 µl	~11.5 to 12.6 µl
Maximum Template Analyzed Volume	~14 µl	~5.5 µl	~7.4 to 8.2 µl

*Based on mean droplet count of 16,000 and measured droplet volume of 0.718 to 0.786 nl[1-2].

The mechanism in which non-analyzed volume can affect sensitivity is by way of introducing subsampling errors. In other words, by not analyzing the whole sample there is a risk that very rare target molecules remain in the dead volume and are not detected. There is also a risk of inaccurate quantification of target molecules with individual aliquots from the same sample source resulting in vast differences in abundance of the target molecules. However, both variables, the template addition volume and the reaction volume analyzed, must be considered to assess their effects on assay sensitivity. We propose combining both factors into a single variable to predict sensitivity, which we call 'Template Analyzed Volume' which is calculated by multiplying the template addition volume by the percentage of the sample that is analyzed by the dPCR instrument (Table 1, Maximum Template Analyzed Volume).

In practice, when the goal of an experiment is to observe rare event targets, the copies transferred (template addition volume) and copies analyzed (template analyzed volume) should be calculated when evaluating different dPCR systems for the experiment. The process of performing rare event detection includes extracting a biological sample which results in a DNA or RNA eluate (Figure 1A). Per the example in Figure 1, a rare event is defined as a template at concentration of ~0.1 to 0.4 copies/µl which results in ~3 to 20 copies in a 30 µl eluate. The number of copies expected to be transferred and to be analyzed across the 3 to 20 copy range are presented in Figure 1. Ultimately, more copies are transferred and analyzed using the QIAcuity digital PCR system compared to the other two technologies, despite their similar or lower non-analyzed volumes. We hypothesis that higher template



		QIAGEN Nanoplate 26k	MAP16 dPCR plate	ddPCR
3 copies in the eluate	Copies transferred	2.60 copies	0.60 copies	1.43 copies
	Copies analyzed	1.40 copies	0.55 copies	0.79 copies
10 copies in the eluate	Copies transferred	8.67 copies	2.00 copies	4.77 copies
	Copies analyzed	4.68 copies	1.82 copies	2.62 copies
20 copies in the eluate	Copies transferred	17.33 copies	4.00 copies	9.53 copies
	Copies analyzed	9.36 copies	3.64 copies	5.24 copies

Figure 1. QIAGEN Nanoplate 26k technology results in higher sensitivity through more copies transferred and analyzed compared to other methods.
Calculations are based on an eluate volume of 30 µl.

analyzed volumes will result in higher sensitivity regardless of dead volume. Additionally, we hypothesize that lower template analyzed volumes will result in higher levels of error (CV) across replicates and higher occurrence of samples containing no target molecules at all. Here we examined the impact of the template subsampling effect by using different template concentrations close to the detection limit at template addition volumes in the dPCR reaction using QIAcuity nanoplates on the QIAcuity instrument.

For comparison with ddPCR system, we used the same template dilutions at the maximal loading volume of 10 µl to perform parallel experiments on the QX200 instrument (Bio-Rad).

Instruments and Materials

Instruments

Instrument	Manufacturer	Cat. No.
QIAcuity Four dPCR system	QIAGEN	911046
Bio-Rad QX200 with Auto DG	Bio-Rad	1864100

Materials

Material	Manufacturer	Cat. No.
Molecular Grade Water	various	various
1x TE Buffer	various	various
QIAcuity Probe PCR Kit	QIAGEN	250103
Custom Primers/Probe mix for QIAcuity (0.8 µM primer and 0.4 µM probe)	custom	N/A
Nanoplate 26k 24-well	QIAGEN	250001
Bio-Rad ddPCR Supermix for Probes	Bio-Rad	1863010
Custom Primers/Probe mix for Bio-Rad (0.9 µM primer and 0.25 µM probe)	custom	N/A
Bio-Rad ddPCR consumables	Bio-Rad	multiple

Templates

Template	Source	Cat. No.
Human genomic DNA from a male donor	prepared internally	n/a
QuantiNova Internal Control DNA (synthetic dsDNA template)	QIAGEN	n/a

Assays

Template	Dye	Source
QN IC	HEX	custom
ERBB2	FAM	custom

Method

We designed the following study to assess the effect of template addition volume, and template analyzed volume. In this study, we generated template solutions that represent the lower bounds of (d)dPCR technologies consisting of 10–40 total copies per 50 µl. We then generated dPCR reactions (Table 2) to assess two template addition volumes, 6 µl per 40 µl reaction and 27 µl per 40 µl. The use of 27 µl template (instead of 26 µl as described in table 1) was enabled by the use of 20x concentrated TaqMan assays. To compare to the ddPCR system which has a different dead volume, we set up reactions using the standard ddPCR assay setup. Each resulting solution was tested in replicate of 12 on the respective system and average concentration, standard deviation, and CV was calculated. Additionally, the number of samples where no positive partitions were observed were counted and the % of negative samples were measured for each set of replicates at each concentration.

Table 2. dPCR reaction setup

Starting template concentration		**Template Addition Volume**	**dPCR reaction volume**	**dPCR system**	**Template Analyzed Volume**	**Expected concentration of dPCR reaction**	**Expected copies per dPCR analyzed**
Total copies	Copies/µl	µl	µl	Name	µl	Copies/µl	Copies
40	0.8	**6**	40	QIAcuity	**3.2**	0.12	2.59
20	0.4	**6**	40	QIAcuity	**3.2**	0.06	1.30
10	0.2	**6**	40	QIAcuity	**3.2**	0.03	0.65
0	0	**6**	40	QIAcuity	**3.2**	0.00	0.00
40	0.8	**27**	40	QIAcuity	**14.6**	0.54	11.67
20	0.4	**27**	40	QIAcuity	**14.6**	0.27	5.83
10	0.2	**27**	40	QIAcuity	**14.6**	0.14	2.92
0	0	**27**	40	QIAcuity	**14.6**	0.00	0.00
40	0.8	**10**	22	QX200	**5.2 to 5.7**	0.36	4.14 to 4.53
20	0.4	**10**	22	QX200	**5.2 to 5.7**	0.18	2.07 to 2.26
10	0.2	**10**	22	QX200	**5.2 to 5.7**	0.09	1.03 to 1.13
0	0	**10**	22	QX200	**5.2 to 5.7**	0	0.00

Results

Our results show that using the QIAcuiy dPCR system, high template addition volumes (27 µl) result in accurate detection of copy numbers (with none-to-little variation between obtained and expected results) across samples with different initial template concentrations (Table 3).

At low template addition volumes (6 µl in QIAcuity and 10 µl in Bio-Rad), however, there is a high deviation between the expected concentrations (in copies/µl) and the measured concentrations. Additionally, at lower template addition volumes we observed an increased uncertainty in copy number estimation, as reflected in higher coefficients of variation (%CV) values across replicates (Table 3 and 4). Furthermore, the percentage of dPCR replicates with no amplification, referred to as negative samples (samples that contain no templates, thus show no positive signals) is significantly higher in samples with low template addition volumes than in samples with high template addition volumes, demonstrating copy number estimation error due to limited template loading amounts.

Subsampling errors become more pronounced at low concentrations when a higher percentage of the partitions are empty, decreasing the accuracy of detection of rare targets in samples. However, subsampling errors become less prominent at higher concentrations or higher template addition volumes, as the likelihood of having target sequences in the PCR reaction increases. Therefore, dPCR systems with high template addition volumes, and thus higher template analyzed volumes, such as QIAcuity, will provide higher sensitivity and accuracy of detection especially for applications such as rare mutation detection, despite the higher dead volume.

Table 3. Results obtained from high and low template addition volumes across various template concentrations on QIAcuity dPCR system

QIAcuity			Concentration (copies /µl)					
			Measured	Expected	StDev of replicates	CV	Number of neg samples	% of neg samples
6 µl volume input	FAM	40 copies/50 µl	0.230	0.120	0.252	**110%**	**0**	**0%**
		20 copies/50 µl	0.103	0.060	0.137	**133%**	**3**	**25%**
		10 copies/50 µl	0.027	0.030	0.028	**105%**	**6**	**50%**
		NTC	0.00	0.00	0.015			
	HEX	40 copies/50 µl	0.12	0.120	0.076	**63%**	**1**	**8%**
		20 copies/50 µl	0.05	0.060	0.056	**105%**	**5**	**42%**
		10 copies/50 µl	0.01	0.030	0.021	**234%**	**10**	**83%**
		NTC	0.00	0.00	0.000			
27 µl volume input	FAM	40 copies/50 µl	0.615	0.540	0.161	**26%**	**0**	**0%**
		20 copies/50 µl	0.336	0.270	0.134	**40%**	**0**	**0%**
		10 copies/50 µl	0.112	0.135	0.063	**56%**	**2**	**17%**
		NTC	0.00	0.00	0.000			
	HEX	40 copies/50 µl	0.51	0.540	0.209	**41%**	**0**	**0%**
		20 copies/50 µl	0.27	0.270	0.117	**44%**	**0**	**0%**
		10 copies/50 µl	0.13	0.135	0.057	**45%**	**0**	**0%**
		NTC	0.00	0.00	0.000			

Table 4. Results obtained from maximum template addition volume across various template concentrations on Bio-Rad ddPCR system

Bio-Rad QX200			Concentration (copies /µl)					
			Measured	Expected	StDev of replicates	CV	Number of neg samples	% of neg samples
10 µl volume input	FAM	40 copies/50 µl	0.272	0.364	0.125	**46%**	**0**	**0%**
		20 copies/50 µl	0.183	0.182	0.098	**54%**	**1**	**8%**
		10 copies/50 µl	0.095	0.091	0.079	**83%**	**3**	**25%**
		NTC	0.03	0.00	0.029			
	HEX	40 copies/50 µl	0.28	0.36	0.162	**58%**	**0**	**0%**
		20 copies/50 µl	0.15	0.18	0.080	**52%**	**0**	**0%**
		10 copies/50 µl	0.08	0.09	0.055	**73%**	**3**	**25%**
		NTC	0.00	0.00	0.000			

Conclusions

Digital PCR is a superior method to qPCR for the detection and absolute quantification of low concentration target templates. There are multiple digital PCR systems on the market that differ in numerous aspects including the amount of dead volume, which is the volume that is loaded but not analyzed by the given instrument. While it has been speculated that dead volume could impact the sensitivity of dPCR applications, here we provide data to support the conclusion that the most important factors in determining the relative sensitivity of each system are template addition volume and template analyzed volume. In summary, data provided herein demonstrate that higher template addition volumes can overcome any limitations that dead volume may have on the sensitivity of a dPCR application.

References

1. Kosir, A.B., et al. Droplet volume variability as a critical factor for accuracy of absolute quantification using droplet digital PCR. *Anal Bioanal Chem* **28**, 6689-6697 (2017).
2. Emslie, K.R., et al. Droplet volume variability and impact on digital PCR copy number concentration measurements. *Anal Chem* **91**, 4124-4131 (2019)
3. Lievens, A., Jacchia, S., Kagkli, D., Savini, C. & Querci, M. Measuring Digital PCR Quality: Performance Parameters and Their Optimization. *Plos One* **11**, e0153317 (2016).
4. The dMIQE Group. The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020. *Clin Chem* **66**, 1012–1029 (2020).

Ordering Information

Product	Contents	Cat. no.
QIAcuity Probe PCR Kit	1 ml 4x concentrated QIAcuity Probe Mastermix, 2 x 1.9 ml Water	250101
QIAcuity Nanoplate 26k 24 well	50 QIAcuity Nanoplates 26x 24-well, 55 Nanoplate Seals	250002
QIAcuity Four Platform System	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit, 1 year warranty on labor, travel, and parts	911042

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