

Effect of Varying Reaction Volumes on Real-Time PCR with the Spartan DX-12™

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Here we demonstrate the effect of reaction volume on Ct precision on the Spartan DX-12™.

Introduction

The unique design of the Spartan DX-12™ instrument enables excitation of reaction mixture from the side of the tube and measurement of emitted fluorescence intensity from the bottom of the tube. As a result of this detection method, the volume of the reaction directly influences the fluorescence output captured by the instrument. With a 20 µl reaction volume, the entire fluorescence capture area is filled. As volume decreases, a smaller proportion of this area is excited, and thus the efficiency of fluorescence capture, and the ability of the instrument to do accurate quantitative measurements, decreases.

The purpose of this study was to determine the effects of differing reaction volumes on real-time PCR repeatability on three Spartan DX-12™ instruments.

Materials and Methods

DNA preparation

A synthetic plasmid construct was designed in-house and produced for Spartan Bioscience by GENEART Inc. (Toronto).

Plasmid DNA was received at a stock concentration of 1.5 mg/ml, reconstituted to 1.5 mM stock in sterile water, aliquoted and stored at -20°C.

Input DNA

Plasmid DNA was diluted to a concentration of 0.75 ng/µl in sterile water.

Real-time PCR

Oligonucleotide primers and probes designed against the Norovirus Open Reading Frame exon 1-2 junction (ORF 1-2) were used (1, 2). The primer & probe sequences are as shown in Table 1.

Components of the Real-Time amplification mixture are listed in Table 3. Samples were amplified in Spartan Tubes (20µl PCR Tube Assembly, Tubes - Cat. No.01004153 and Caps - Cat. No. 01004155), and amplification was performed on the Spartan DX-12 instrument. Table 2 shows the cycling parameters used (2-temperature program).

DNA analysis

Fluorescence data was transferred from the Spartan DX-12 and graphed using the Spartan Graphing & Analysis Software (ver. 3.10).

Primer/Probe	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
COG1 primers	CgYTgg ATg CgN TTY CAT gA	CTT AgA CgC CAT CAT CAT TYA C	85
COG1 probe	6-FAM-AgA TYg CgR TCY CCT gTC CA g-BHQ1		
COG2 primers	CAR gAR BCN ATg TTY AgR Tgg ATg Ag	TCg ACg CCA TCT TCA TTC ACA	98
COG2 probe	CAL Fluor® Red 610 -Tgg gAg ggC gAT CgC AAT CT-BHQ2		

6-FAM = 6-carboxy-fluorescein, BHQ = Black Hole Quencher

Table 1. Primer/probe sequences and amplicon sizes.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	30 s	1
Denaturation	95°C	25 s	40
Annealing/extension	55°C	25 s	40

Table 2. Cycling parameters.

Results

Figure 1 shows representative plots of normalized fluorescence across 12 wells, for both the green and the red channels. Here we see there is a significant variation in Ct values across all wells at lower reaction volumes (particularly 5 µl), which improves as the volume increases to 20 µl, which corresponds to the volume of reaction excited, shown in Table 4.

Tables 5 (green channel) and 6 (red channel) show the average Ct values, standard deviation and maximum delta Ct values

Component	Final amount
10X PCR Reaction Buffer (No MgCl ₂) (Invitrogen)	1 X
MgCl ₂ (Invitrogen)	2.5 mM
dNTP mix (Invitrogen)	0.125 mM
Taq DNA polymerase (Invitrogen)	1 U
Probes (Biosearch Technologies)	0.2 μM (COG1), 0.05 μM (COG2)
PCR primers (IDT)	0.2 μM (each)
Template DNA	2 μl
Sterile water	up to 5-20 μl
Total reaction volume	5-20 μl

Table 3. Components of PCR amplification mixture.

across 12 wells, on three different instruments. Although the average Ct values are consistent between volumes of 10-20 μl (results vary from 10.84-10.03, with standard deviation values varying from 0.66-0.26 in green, and 9.86-9.04 with standard deviation values of 0.69-0.22 in red), results at 5 μl are inconsistent. Maximum deltas between Ct values observed are: 10.9 and 4.1 at 5 μl; 2.3, and 1.7 at 10 μl; 1.3 and 1.4 at 15 μl;

Total Volume	Volume Excited	% Maximum Fluorescence
20 μl	13.6 μl	100
15 μl	13.6 μl	96
10 μl	8.6 μl	63
5 μl	3.5 μl	26

Table 4. Amount of total fluorescence excited relative to sample volume.

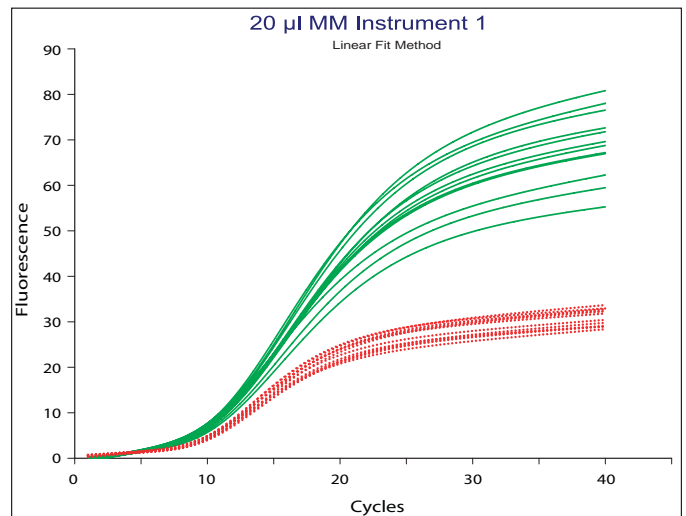
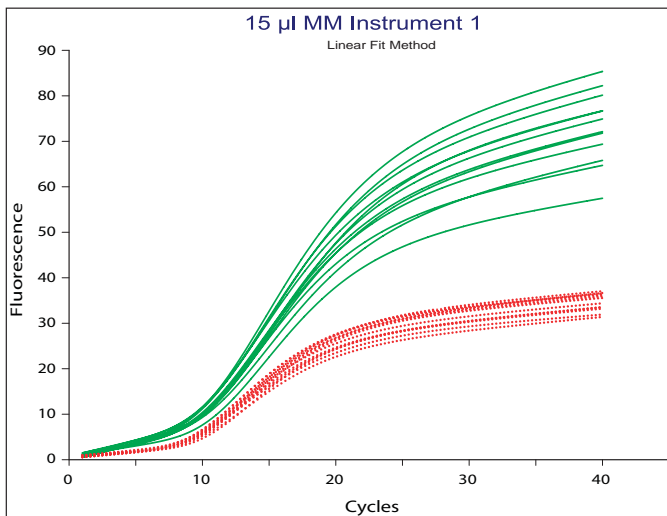
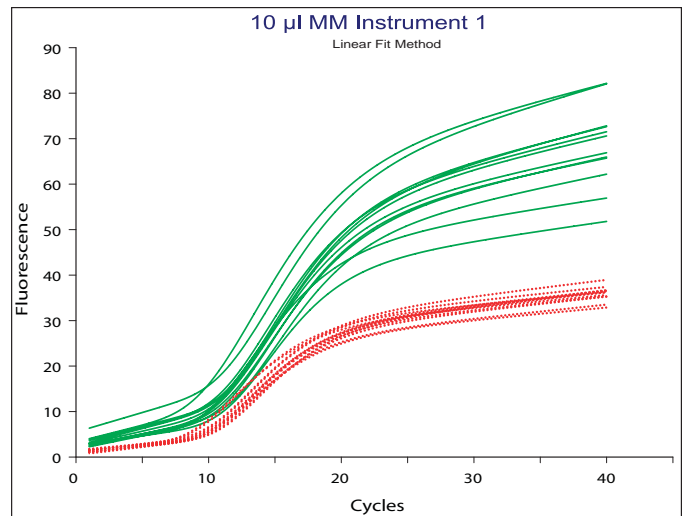
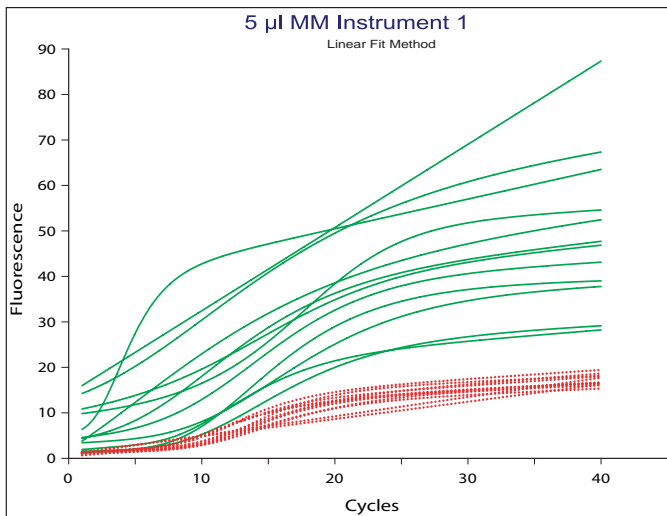


Figure 1. Real-Time PCR results for 12 wells of 5 μl, 10 μl, 15 μl & 20 μl reaction volume repeatability on the Spartan DX-12™ (Instrument 1).

Volume (µl)	Instrument	Average Ct	Standard Deviation	Max ΔCt
20	1	10.63	0.29	1
	2	10.66	0.26	0.8
	3	10.83	0.34	1
15	1	10.13	0.27	1.3
	2	10.12	0.41	1.3
	3	10.03	0.43	0.8
10	1	10.84	0.69	2.3
	2	9.23	0.57	2
	3	10.36	0.57	1.7
5	1	9.5	1.74	5.3
	2	8.04	3.77	10.9
	3	7.22	3.37	10.7

Table 5. Average Ct, Standard deviation and maximum delta Ct observed across 12 wells of Real-Time PCR, in the green channel, using reaction volumes of 5 µl, 10 µl, 15 µl, and 20 µl.

Volume (µl)	Instrument	Average Ct	Standard Deviation	Max ΔCt
20	1	9.69	0.22	1
	2	9.58	0.27	0.9
	3	9.57	0.32	0.7
15	1	9.23	0.37	1.4
	2	9.04	0.44	1.4
	3	9.29	0.43	1.2
10	1	9.86	0.69	2.3
	2	8.47	0.66	2.3
	3	9.58	0.57	2.2
5	1	9.44	1.17	4.1
	2	9.78	0.89	3
	3	9.13	1.04	3.4

Table 6. Average Ct, Standard deviation and maximum delta Ct observed across 12 wells of Real-Time PCR, in the red channel, using reaction volumes of 5 µl, 10 µl, 15 µl, and 20 µl.

and, 1 and 1 at 20 µl (for green and red respectively).

Discussion and Conclusions

The results demonstrate that the Spartan DX-12™ can be used for volumes as low as 10 µl for plus/minus applications, however 20 µl reaction volumes are recommended for applications requiring any quantitative analysis.

References

- Rolf KJ *et al.* (2007). An internally controlled, one-step, real-time RT-PCR assay for norovirus detection and genogrouping. *Journal of Clinical Virology*. 39(4): 318-21.
- Kageyama T *et al.* (2003). Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology*. 41(4): 1548-57.

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