

Assay Assembly for Miniaturized Quantitative PCR in a 384-well Format Using the Echo Liquid Handler

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Introduction

Quantitative PCR (qPCR) has enabled a wide range of real-time applications including comparisons in gene expression, genotyping and SNP analysis. Reductions in scale reduce sample and reagent volumes and therefore total running and operational costs. To ensure high data quality, the liquid handling employed in low volume reactions must be exceptionally precise and accurate. This application note highlights the capabilities of the Echo liquid handler to dispense into 384-well qPCR plates with total reaction volumes as low as 250 nanoliters. Standard deviations of less than 0.25 and CVs of less than 2% are seen routinely across plates using the low volume dispensing of the Echo liquid handler. The ability of the Echo liquid handler to transfer from any well to any well simplifies assay setup. The results demonstrate the capabilities to enable assay setup with flexible plate layouts, reduce reagent volumes and increase throughput.

Experiment 1: Quantitative PCR miniaturization in 384-well format

To evaluate the ability to miniaturize qPCR experiments, 250 to 2000 nanoliters of a qPCR reaction mix was transferred from a 384-well Echo source plate (Labcyte Inc.) into a 384-well qPCR plate (Roche Applied Science). Resulting amplification values were compared.

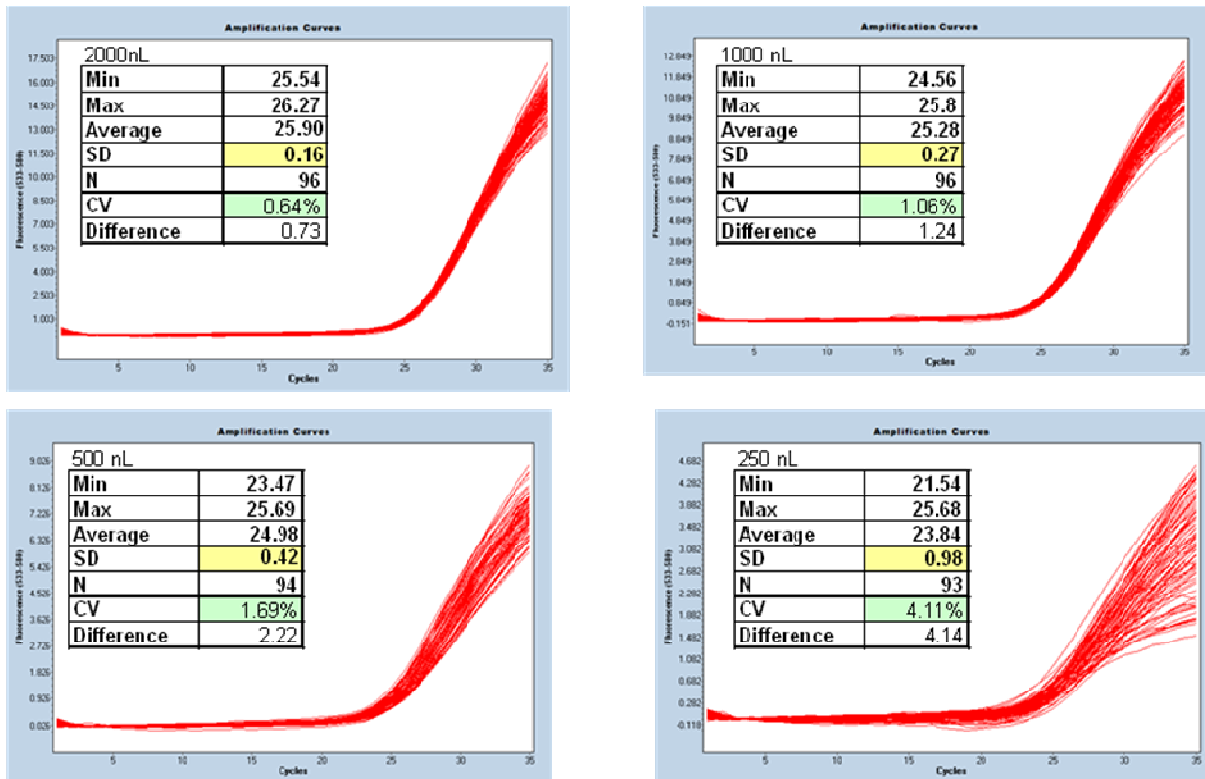
Methods

Pre-mixed PCR solution containing 1 ng/μL of cDNA (Clontech), 600 nM GAPDH primers (UPL, Roche Applied Science), 400 nM GAPDH probe (UPL, Roche Applied Science), and 1X master mix (Real Time Ready Probes Master, Roche Applied Science) was dispensed into 12 wells of a 384-well Echo qualified polypropylene source plate (Labcyte Inc). The Echo 555 liquid handler was then used to transfer 250, 500 and 1000, and 2000 nL of the reaction mix to each quadrant of a 384-well qPCR plate (Roche Applied Science). Reactions were thermal cycled in a LightCycler® 480 System (Roche Applied Science). Thermal cycle conditions were as follows: 95°C for 60 seconds, followed by 45 cycles of 95°C for 0 seconds and 60°C for 60 seconds. Amplification results were quantified using the LightCycler 480 system software. Each quadrant of the plate represents a different amount and thus different end-point signal output.

Results

Resulting data demonstrated an increasing endpoint signal which directly corresponded to the total reaction volume in the plate (Figure 1). Excellent standard deviations for 2000 nL and 1000 nL volumes were 0.16 and 0.26, respectively, with coefficients of variation (CV) of 0.64% and 1.06%, respectively. Decreased volume below 1000 nL resulted in higher standard deviation and CVs, with a maximum of 4% for a 250 nL reaction volume.

Figure 1. Cq (Quantification Cycle) Graphs for low-volume qPCR



Experiment 2: cDNA dilution series using the Echo liquid handler for 384-well qPCR

To evaluate the ability of the Echo liquid handler to directly dilute cDNA without pre-mixed dilution steps, increasing volumes of concentrated stock cDNA were transferred into a 384-well qPCR destination plate to create a series dilution.

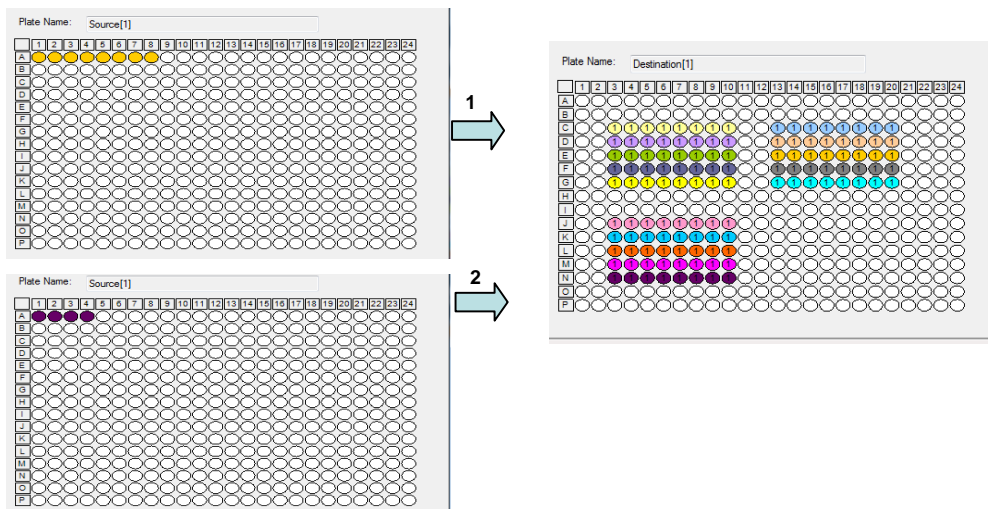
Methods

Using a two-step process, the Echo liquid handler was used to dispense master mix with premixed primers and probes into a qPCR plate, followed by incremental transfer of stock human reference cDNA. Echo Plate Reformat software was used to setup the transfer of master mix and cDNA, see Figure 2 below. Source plates containing 50 µL of premixed master mix, primers and probes (600nM and 400nM GAPDH respectively) were pipetted into eight wells of an Echo 384-well polypropylene source plate. A protocol was created using Echo Plate Reformat to dispense incremental fill volumes of master mix (see Table 1). A second source plate (low dead volume, Echo qualified) containing human reference cDNA at a concentration of 50ng/µL was used to create the dilution series. Echo Plate Reformat software was setup to transfer a four-fold dilution series from 2.5 nL to 640 nL. After the protocols were created, the actual dispense time for both plates totaled less than 8 minutes.

Table 1.

Dispense volumes for cDNA dilution series qPCR					
Final cDNA concentration	0.13 ng	0.5 ng	2.0 ng	8.0 ng	32.0 ng
Master mix (nL)	2000	2000	2000	1840	1360
cDNA (nL)	2.5	10	40	160	640
Total	2002.5	2010	2040	2000	2000

Figure 2. Echo Plate Reformat software plate maps for cDNA dilution series qPCR

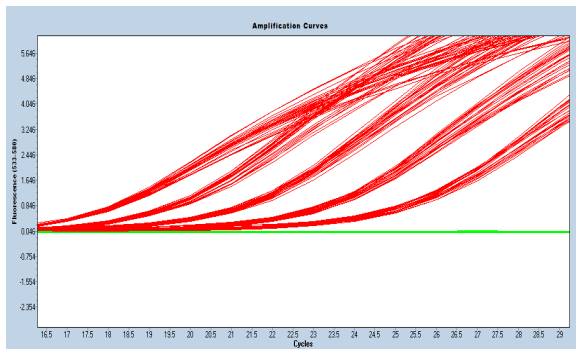


1. Master mix containing primers and probes at 1X concentration.
2. Stock cDNA at 50 ng/μL.

Results

Resulting amplification curves demonstrated a distinct delineation between concentration and cycle quantification (Cq) value for each change in concentration (Figure 3). Data analysis for each of the 24 replicates showed excellent standard deviations from 0.085-0.18 with CVs of the Cq ranging from 0.50-0.73%.

Figure 3. Cq results and corresponding data analysis for a qPCR cDNA dilution series



0.13 ng cDNA (2.5 nL)		0.5 ng cDNA (10 nL)		2.0 ng cDNA (40 nL)		8 ng cDNA (160 nL)		32.0 ng cDNA (640 nL)	
Cq Min	24.66	Cq Min	22.84	Cq Min	20.83	Cq Min	19.11	Cq Min	17.05
Cq Max	25.63	Cq Max	23.34	Cq Max	21.48	Cq Max	19.63	Cq Max	17.33
Cq Average	25.11	Cq Average	23.09	Cq Average	21.15	Cq Average	19.31	Cq Average	17.17
SD	0.18	SD	0.13	SD	0.17	SD	0.15	SD	0.09
N	24	N	24	N	24	N	24	N	24
CV	0.73%	CV	0.55%	CV	0.80%	CV	0.80%	CV	0.50%
Difference	0.97	Difference	0.50	Difference	0.65	Difference	0.52	Difference	0.28

Experiment 3: qPCR assay assembly using Echo Plate Reformat software

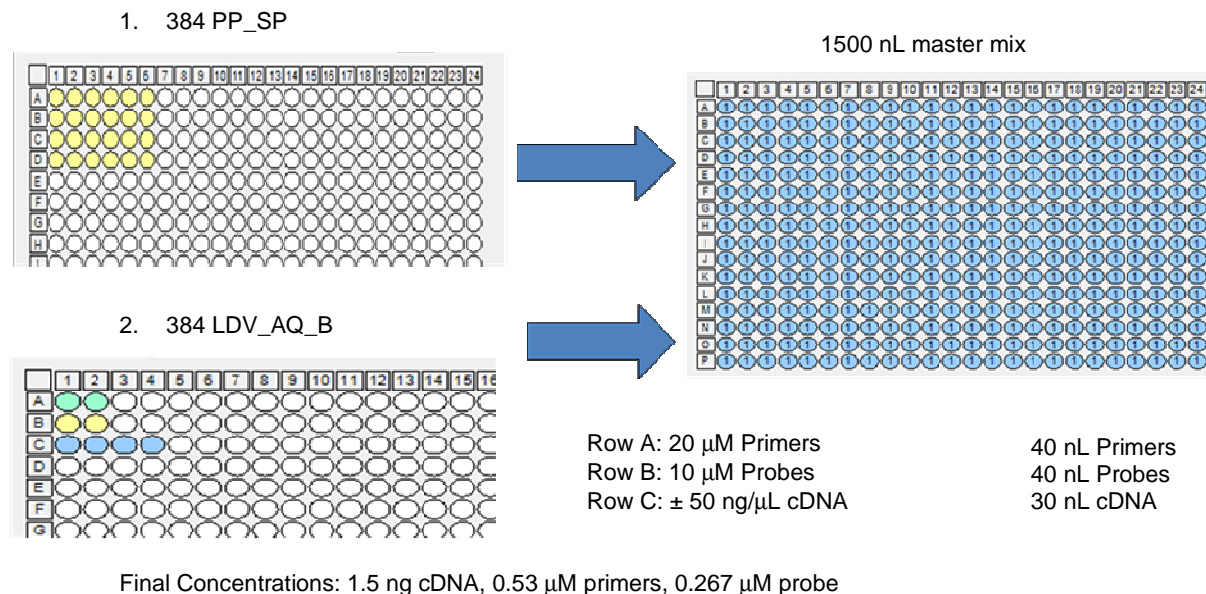
The Echo liquid handler was used to generate a complete qPCR from individual components.

Methods

Two polypropylene source plates were used; one to dispense the master mix, and a second low dead volume plate to dispense primers, probes, and cDNA. First, 1500 nL of a 1X master mix was transferred to the 384-well qPCR plate. This was followed by 40 nL of primers and 40 nL of probe. Thirty nL of alternating cDNA or water controls were added, as shown in Figure 4. In this experiment, alternating amounts of water and of cDNA were dispensed to mimic zero cross contamination

experimental conditions. Concentrations of stock solution were 20 μM GAPDH primers, GAPDH 10 μM , and 50 $\text{ng}/\mu\text{L}$ of human reference cDNA.

Figure 4. Plate Reformat software setup for multi-step qPCR reagent transfer

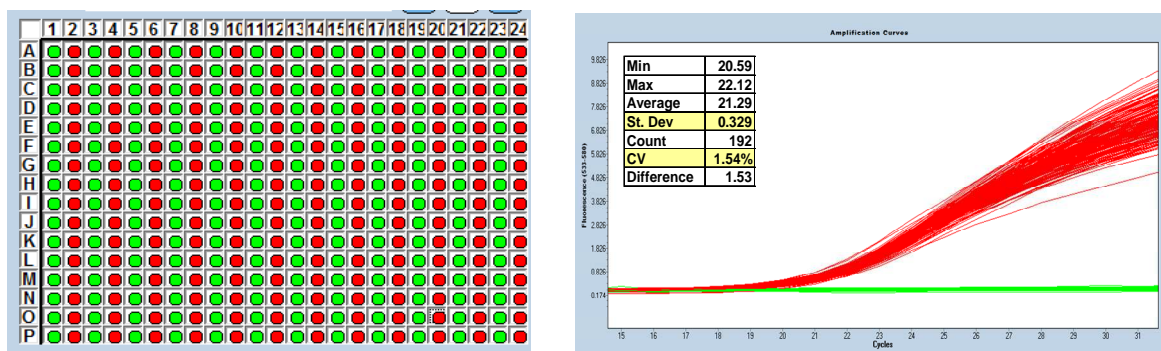


Results

The heat map results displayed in Figure 5 show the positive and negative calls from the software analysis of the Cq values. The positive calls are shown in green and the negative calls are shown in red. The precise non-contact transfer of the Echo liquid handler enabled zero cross contamination despite a four step dispense. Figure 6 shows the amplification results as well as the standard deviation and CVs for 192 positive samples. The standard deviation was 0.32 with a CV of 1.54% for a 1500 nL final volume.

Figure 5. Heatmap results of qPCR assay assembly

Figure 6. Amplification results of qPCR assay assembly



Summary

The Labcyte Echo liquid handling platform presents exciting new capabilities for total assembly and miniaturization of qPCR in 384-well formats. The ability of the Echo liquid handler to transfer from any well to any well enables experiment assembly utilizing concentrated stock material without the typical pre-pipetting steps required to dilute source solutions. Positional accuracy allows accurate transfer without causing cross contamination. Superior volumetric precision and accuracy ensure excellent cycle quantification precision even with very little target DNA in very low reaction volumes. This technology enables scientists to fully explore the capabilities of miniaturized PCR and other genomics applications, while reducing reagent consumption and therefore operational running costs.