Plate Preparation for the CFX384™ Real-Time PCR Detection System Using a Tecan Robotics System

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Introduction

High-throughput real-time PCR data collection is becoming increasingly important because screening a large number of samples is often needed to obtain meaningful results. Additionally, to reduce the overall cost of assays, the use of smaller reaction volumes is being explored. Changing from the standard 96-well format to a 384-well format greatly increases capacity; however, the transition can be challenging because of the smaller well size and reduced volumes. To pipet reaction mixtures manually, and still obtain consistent results, requires a considerable amount of skill and can be quite tedious. An alternative to manual pipetting is to aliquot reaction mixes using an automated liquid handling system.

In this work, we demonstrate the ability of the Freedom EVO® 150 liquid handling system (Tecan Group Ltd.) to reliably aliquot reaction mixes for the generation of qPCR data on Bio-Rad's CFX384 real-time PCR detection system. Both uniformity and linearity assays were performed. In the uniformity assay, the same reaction mix was dispensed into all wells. An acceptable result is one in which amplification occurs at approximately the same cycle for all wells (±0.2 quantification cycle [Cq] standard deviation). In the linearity assay, template was serially diluted tenfold from 1 x 10^7 to 1 x 10^2 to produce a standard curve, which would, ideally, have a reaction efficiency of 95–105%.

The results of the uniformity and the linearity assays (low standard deviation and optimal reaction efficiencies, respectively) demonstrate that the combination of the Freedom EVO 150 liquid handling system and the CFX384 real-time PCR detection system generates consistent and reproducible high-throughput, low-volume, real-time qPCR data.

Methods

Real-Time PCR System and Reagents Used

The CFX384 real-time PCR detection system, iQ™ supermix, Hard-Shell® 384-well skirted PCR plates, and Microseal® 'B' adhesive seals were from Bio-Rad Laboratories, Inc.

The following template, primers, and probe were used:

- IL-1β plasmid (I.M.A.G.E. Consortium clone ID 324655, American Type Culture Collection): 10^5 copies/µl for the uniformity studies and 10^7 copies/µl starting concentration for the linearity studies
- IL-1β forward primer: 5' TGC TCC TTC CAG GAC CT 3' (Integrated DNA Technologies, Inc.)
- IL-1β reverse primer: 5' GTG GTG GTC GGA GAT TC 3' (Integrated DNA Technologies, Inc.)
- IL-1β FAM probe: 5' FAM-CTCTGCCCTCTGGATGGCG-Black Hole Quencher 1 (BHQ-1) 3' (TriLink BioTechnologies, Inc.)

Real-Time PCR Protocol

The protocol used for amplification was 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and 55°C for 30 sec, then a plate read.

General Considerations for a Liquid Handling Robotics Setup

- The deck layout should be the most logical to reduce time and avoid transferring samples directly above open stock tubes
- The liquid handling system should be initialized and primed
- Supermix should be gently mixed by inverting, reagents vortexed, and all tubes briefly centrifuged
- Ideally, liquid level sensing should be active. All aspirations and dispenses should occur at the liquid level surface. Dispensing into empty vessels should occur as close to the bottom of the vessel as possible and be automatically adjusted with the liquid surface

Liquid Handling Robotics System Hardware

- Freedom EVO 150 system with an 8-channel liquid handling arm (LiHa)
- 500 µl syringes; disposable tip adaptors with low tip eject option; 10, 50, and 200 µl filtered Tecan conductive disposable tips
- Carriers on the worktable included:
  - 4-position disposable tip (DiTi) carrier with 3 positions for tips and 1 position for disposable tip waste
  - Standard wash station
  - 384-well–compatible microplate carrier
  - Tube carriers for 1.5, 2.0, and 0.5 ml tubes

Liquid Handling Robotics System Software

- Freedom EVOware® software version 2.3 or higher
- A Freedom EVOware export file has been prepared that contains the liquid classes used during testing. Contact Tecan at applications.solutions@tecan.com for more information

Liquid Handling Robotics System Protocols

- The scripts used as a template for starting can be obtained by contacting the authors
- The scripts were modified to accommodate the various volumes used

Liquid Handling Robotics System Procedures for All Scripts

- Volumes were adjusted for 500 µl syringes and the largest disposable tips volume of 200 µl. For example, if the aspiration volume was 600 µl, then 3 x 200 µl was used
- Additional mixing steps were added to ensure uniformity of master mix solutions and serial dilution standards. This included mixing at different heights in the tubes and dispensing at liquid level with mixing
- Appropriate wash steps were inserted to maintain good liquid handling. Additionally, the air gaps were reset
- Dispensing into the 384-well plate was done with disposable tips very near the bottom of the well. For the small volumes of 3 and 5 µl, wick or touch off was used. Volumes below 5 µl produce drops that are too small to consistently dislodge from tips using air dispensing

Liquid Handling Robotics System Modifications for Specific Scripts

Uniformity assay at 3 or 5 µl per well:

- When making master mix, an additional mixing step was added after dispensing each reagent aliquot and before transfer to the 384-well plate
- Multidispensing into the 384-well plate
  - — Excess master mix was made to ensure that every well received 3 or 5 µl
  - — Foaming was minimized by using optimized liquid handling parameters
  - — 8 x 3 µl (or 8 x 5 µl) was dispensed with 50 µl tips

Linearity assay at 5 or 10 µl per well:

- When making master mix, an additional mixing step was added after dispensing each reagent aliquot
- For plasmid serial dilutions, an additional mixing step was added after dispensing plasmid
- Multidispensing into the 384-well plate
  - — 8 x 5 µl was dispensed with 50 µl tips for each plasmid concentration
  - — 8 x 10 µl was dispensed with 200 µl tips for each plasmid concentration

Results

Uniformity Assay

FAM-labeled hydrolysis probe reaction mix (3 µl) was aliquoted into all the wells of the 384-well plate. As shown in Figure 1, at the lowest volume analyzed (3 µl), all wells exhibited a Cq value of 19.1 ± 0.157, which is within acceptable limits for the standard deviation (<0.2). When the 5 µl reaction set was run, the Cq was 18.2 ± 0.132 (data not shown).

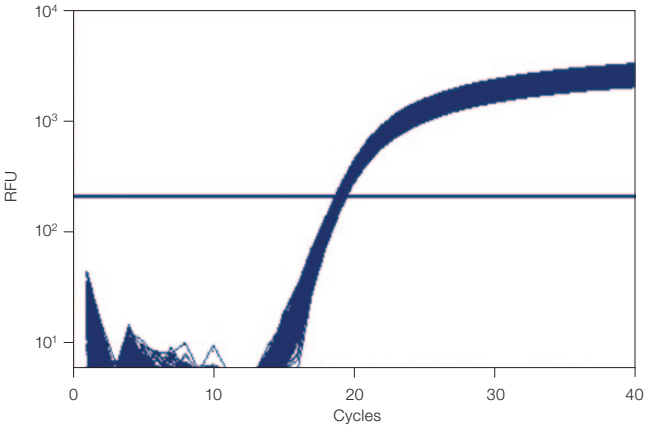

Fig. 1. Uniformity assay using 3 µl reaction volume. The same reaction mix (containing a FAM-labeled hydrolysis probe) was dispensed into all wells. Cq standard deviation = 0.157. RFU, relative fluorescence units.

Linearity Assay

For both 5 µl (Figure 2) and 10 µl (Figure 3) reactions, when template was serially diluted tenfold, the resultant standard curves were in the ideal range (95–105%), as were the coefficients of determination (R^2 >0.980).

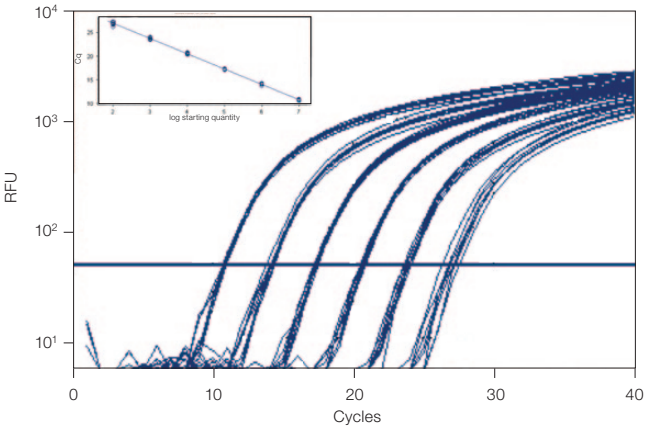

Fig. 2. Linearity assay using 5 µl reaction volume. Template was serially diluted tenfold with eight replicates each. Inset shows the standard curve. Efficiency = 103.7%, R^2 = 0.999. Cq, quantification cycle; RFU, relative fluorescence units.

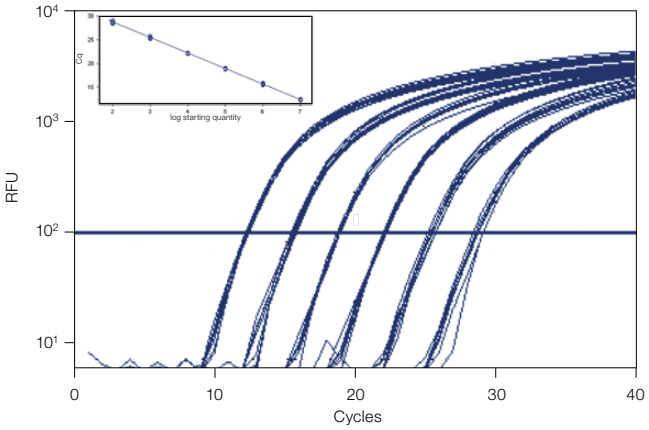

Fig. 3. Linearity assay using 10 µl reaction volume. Template was serially diluted tenfold with eight replicates each. Inset shows the standard curve. Efficiency = 101.5%, R^2= 0.999. Cq, quantification cycle; RFU, relative fluorescence units.

Conclusions

As reaction volumes and well sizes decrease and the number of samples that laboratories process increases, it can become a challenge to manually dispense reaction mixes and obtain consistent, reliable data. The results presented in this tech note demonstrate that the Freedom EVO 150 liquid handling robotics system can be easily utilized with the CFX384 real-time PCR detection system to produce 384-well plates containing low-volume reaction mixes that yield high-quality, uniform qPCR data.

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