Sample Integrity in qPCR: Using PIPETMAX® qPCR Assistant to Minimize Contamination

APPLICATION NOTE TRANS0116

Cross-contamination is a common concern when a laboratory contemplates moving towards automating an assay. Quantitative-PCR detects minute amounts of DNA, making it a technique that is extremely sensitive to cross-contamination and carryover. For this reason, sample integrity is crucial. A sample that has been contaminated may produce a false positive, and thereby negatively impacting critical research or a clinical analysis. The inclusion of proper controls, such as positive and negative, helps to avoid confusion late in the analysis process. While positive and negative controls are valuable, clean sample processing is also useful. The implementation of automated sample processing can further increase confidence in data. The PIPETMAX® with qPCR Assistant is a highly proficient pipetting workstation that reduces the potential for cross-contamination and other human induced errors.

INTRODUCTION

Quantitative PCR (qPCR) is process that is fraught with the potential for contamination, either from the environment or through cross-contamination during the reaction setup process. Often, if contamination is discovered within an assay, it is recommended to dispose of all reagents that were used to prepare the reactions, as identifying the source of the contamination may be more time consuming and costly than discarding and starting over.

Figure 1
PIPETMAX with qPCR Assistant is an automated pipetting workstation that can be used to simplify qPCR reaction setup.

For this reason, safe-guards may be enacted that ensure a single flow of physical materials in the lab, such as samples being processed in a separate environment from where the reaction mixes are prepared, and even a third area for combining the sample dilutions and the reaction mix.

Furthermore, the inclusion of proper controls in the qPCR assay ensure that contamination is quickly detected. No template controls (NTC), negative control of extraction (NCE), and environmental controls (EC) may be used to identify contamination and the potential source. The use of an automated pipetting workstation, such as PIPETMAX (Figure 1) can reduce the potential for cross-contamination by reducing the risk of human error. PIPETMAX was used to generate three qPCR plates, with multiple assays (targets), and samples in three different plate layouts (speed, organized, and random).

MATERIALS AND METHODS

Samples

DNA was isolated from plant extracts (grapevine, apple, pear, and peach) and tested for the presence of phytoplasmas by qPCR with universal assay[1] and with phytoplasma specific assays[2,3] (Table 1).

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Table 1
Six different qPCR assays were used to detect various fruit tree specific phytoplasmas.

Assay	Phytoplasma/disease	Host
BN	Bois noir	Grapevine
FD	Flavescence dorée	Grapevine
AP	Apple Proliferation	Apple
PD	Pear decline	Pear
ESFY	European stone fruit yellow	Peach
Universal	All tested phytoplasmas	Different hosts

DNA Extraction

DNA was extracted from grapevine (4), apple (2), pear (2) and peach (2) samples using previously established methods[4]. Cellular disruption was achieved using a FastPrep®24 (MP Biomedicals) instrument. DNA was extracted with the QuickPick™ SML Plant DNA kit (Bio-Nobile).

Automated qPCR Setup

Real-time PCR reactions were created with the Gilson qPCR Assistant and PIPETMAX automated pipetting workstation. A sample list was imported into the qPCR Assistant software, which then created the automated protocols that were run on PIPETMAX. Three different plate layouts were chosen (Figure 2) to illustrate the capabilities of the system and the flexibility to fit into laboratory workflows.

A

Figure 2
qPCR Assistant generated plate layouts for automated sample preparation on the PIPETMAX. A.) Plate formatted for optimal pipetting with a multichannel system (speed mode).

B

Figure 3
qPCR Assistant generated plate layouts for automated sample preparation on the PIPETMAX. B.) Reactions assigned random locations throughout the reaction plate (randomize plate).

A manufacturer-specific thermal cycler import file is generated by the qPCR Assistant. This file contains all of the reaction information, such as sample names, reporters, targets, dilutions, etc., and can be imported directly into the thermal cycler, thereby eliminating the time required for data input and also the possibility of input error. qPCR Assistant supports three different plate layout options: Speed, Organization, and Randomize. The plate layout format may be selected based on the needs of the researcher:

- **Speed:** This format is used when a thermal cycler can easily manage the information provided by the qPCR Assistant thermal cycler file. The resulting plate layout maximizes multichannel pipette tip usage when dispensing onto the reaction plate. This will be the fastest protocol to complete.
- **Organization:** This format will organize a plate according to the assay being performed, and will keep samples and controls for a given assay together. This protocol may take longer to perform, but will simplify data analysis in the thermal cycler software.
- **Randomize:** This format will randomly place reactions throughout the reaction plate. While not commonly used, randomizing a plate provides a quality check, ensuring that no artifacts exist from the thermal cycler.

Real-time PCR

The phytoplasma assays (BN, FD, AP, PD, ESFY, and Universal) were set up according to previously established methods[1,2,3,4,5]. The FD and BN assays detect the grapevine phytoplasmas causing flavescence dorée and bois noir respectively. The AP, PD, and ESFY assays detect specific phytoplasmas

causing diseases on apple (apple proliferation), pear (pear decline), and prunus species (European stone fruit yellow) respectively. The Universal assay[1], which detects all tested phytoplasmas, was performed on all samples. Each assay was set up in triplicate with positive (PC) and negative controls (NTC). Thermal cycling and detection were completed on a 7900HT Fast Real-Time PCR System (Applied Biosystems™) in optical 384-well plates using universal cycling conditions (2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C).

RESULTS AND DISCUSSION

For each sample and control (PC and NTC), the results were as expected. All of the negative controls (NTC) gave no response, while all of the positive controls (PC) produced appropriate signals. Each experiment had a sample that produced a positive detection and one that produced a negative detection with specific assay. In each case, the Universal assay had a matching result (Figures 3–6). The combined average Cq value from the three different automated pipetting modes (Speed, Organization, Randomize), each in triplicate (n=9) is shown. The %CV for each sample was less than 0.84% across the three different modes, with no carryover or cross- contamination between the samples and standards, regardless of processing mode.

The sample Grapevine-1 was positive for FD phytoplasma, while Grapevine-2 was positive for BN phytoplasma, but not FD. In Grapevine-3 mixed infection with both phytoplasmas (FD and BN) was detected. No phytoplasma was detected in Grapevine-4 (Figure 3). The sample Apple-2 was shown to be positive for the phytoplasma causing AP, while no phytoplasma was detected in Apple-1 (Figure 4). The sample Pear-2 was shown to be positive for the phytoplasma causing PD, while no phytoplasma was detected in Pear-1 (Figure 5). The sample Peach-2 was shown to be positive for the phytoplasma causing ESFY, while no phytoplasma was detected in Peach-1 sample (Figure 6).

The results showed that PIPETMAX with qPCR Assistant can be used to easily automate a process that is prone to error. No carryover or cross-contamination was observed, despite having positive and negative samples and controls side by side in a 384-well format. The data were generated in three different qPCR runs, utilizing three different plate layout options (Speed, Organization, Randomize), demonstrating that PIPETMAX can be used to increase assay confidence by ensuring sample integrity. The %CV for each sample ranged from 0.16%–0.84%, thereby demonstrating high precision.

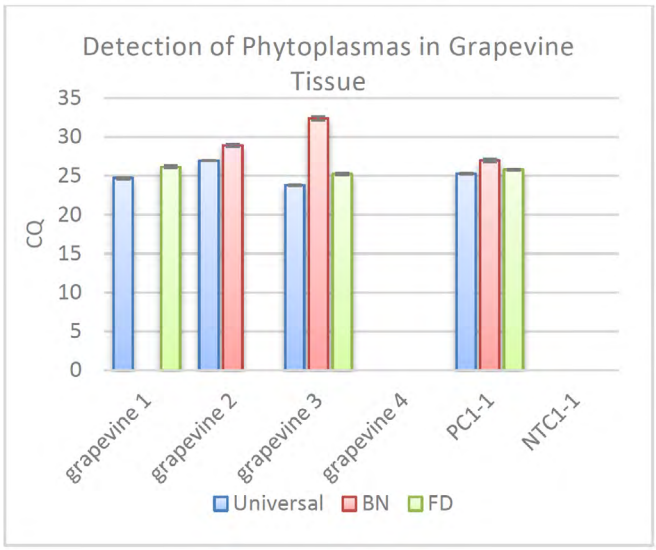

Figure 4
Detection of grapevine phytoplasmas in four grapevine samples via the BN, FD, and Universal assays. Grapevine-1, -2, and -3 showed positive detection of at least one phytoplasma, while Grapevine-4 was found to be free of phytoplasmas disease.

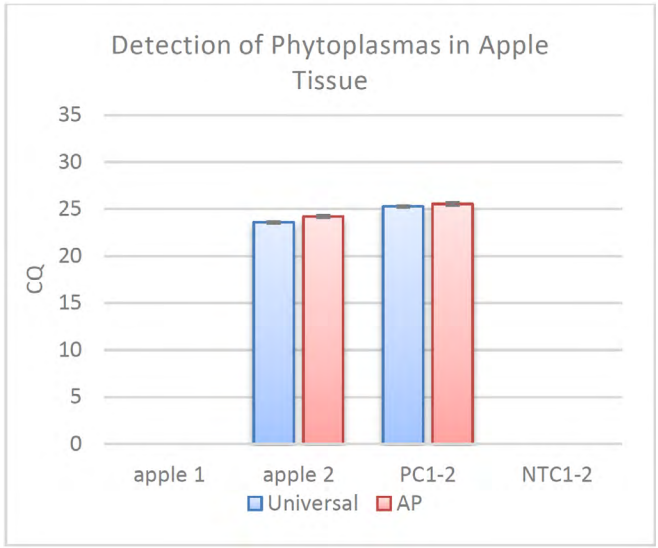

Figure 5
Detection of phytoplasmas causing AP in two apple samples via the AP and Universal assays. Apple-2 showed positive detection of a phytoplasma in both the AP and Universal assay, while Apple-1 was found to be free of phytoplasmas disease.

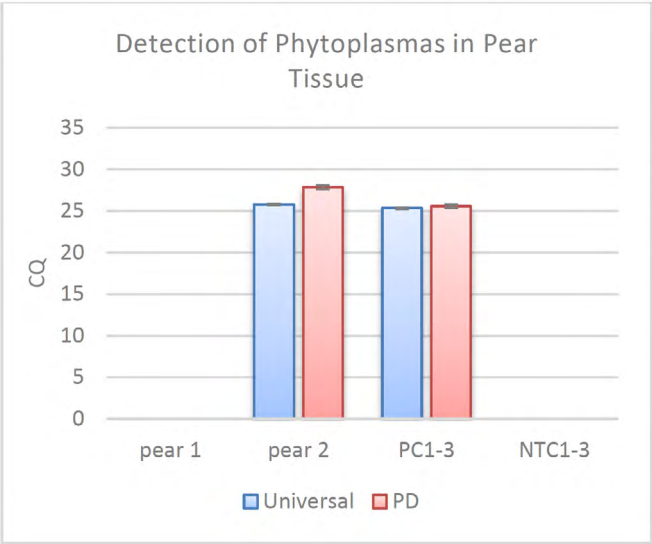

Figure 6
Detection of phytoplasmas causing PD in two pear samples via the PD and Universal assays. Pear-2 showed positive detection of a phytoplasma in both the PD and Universal assay, while Pear-1 was found to be free of phytoplasmas disease.

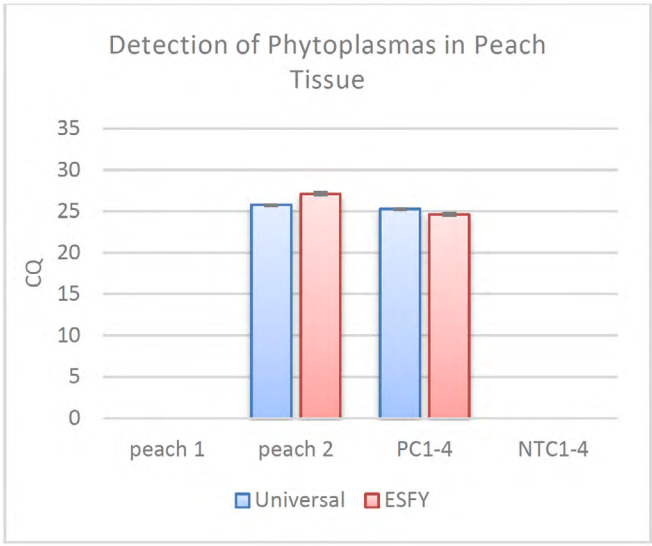

Figure 7
Detection of phytoplasmas causing ESFY in two peach samples via the ESFY and Universal assays. Peach-2 showed positive detection of a phytoplasma in both the ESFY and Universal assay, while Peach-1 was found to be free of phytoplasmas disease.

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CONCLUSIONS

- PIPETMAX with qPCR Assistant easily automates a technique that is prone to error.
- The data were collected using three different plate layout options, and demonstrates the reliability of the automated process.
- No carryover or cross-contamination was observed, despite having positive and negative samples and controls side-by-side in a 384-well format.