# PIPETMAX: Automating 384-Well qPCR Plate Preparation

**APPLICATION NOTE AN0990**

### APPLICATION BENEFITS

Gene expression studies are often used to gain insight into complex interactions between organisms, including those between plants and viruses. Real-time PCR (qPCR) is a tool frequently used in these studies given its wide dynamic range, sensitivity, and ease of automation.

### SOLUTIONS

Gilson's PIPETMAX® can be used to automate qPCR sample preparation prior to measuring relative gene expression. Furthermore, statistical analysis of the qPCR results demonstrates the ability of PIPETMAX® to automate sample preparation accurately, eliminate variability, and do so without introducing contamination.

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## INTRODUCTION

Plant-virus interactions are an example of very complex interactions best understood by monitoring gene expression of host plants after infection with a virus.[1] In this case, we monitored the response of the potato plant cultivar Désirée (*Solanum tuberosum L. cv.* Désirée) to infection with Potato Virus Y (PVY) at the level of gene expression. Potato is the world's most widely grown tuber crop, and the fourth largest food crop in terms of fresh produce after rice, wheat, and corn. This makes PVY, a member of the *Potyviridae* family, an important potato pathogen worldwide.[2] Désirée is a tolerant cultivar, which means that PVY viruses multiply in the plant, but the plant tolerates PVY infection with very mild or no symptoms.

In this application, the PIPETMAX® (Figure 1) was used to automatically prepare 384-well qPCR plates in order to monitor PVY levels post infection.

The discussion describes the results of the relative gene expression of the Chlorophyll a/b binding gene (CAB), and PVY RNA linear regression values from serial dilutions, cytochrome oxidase (COX) gene amplification curves, and statistical analysis of pipetting (%CV).

**Figure 1**
PIPETMAX®

## MATERIALS AND METHODS

### Materials

- RNeasy Plant Mini Kit - Qiagen, Hilden, Germany
- DNase I - Invitrogen, Carlsbad, CA
- High-Capacity cDNA Reverse Transcription Kit - Applied Biosystems, Carlsbad, CA
- 7900HT Fast Real-Time PCR System with Fast 384-Well Block Module.

### Sample Preparation

1. Two plants were inoculated with PVY, and two were mock-inoculated. Leaves from virus- and mock-inoculated plants were sampled at 4 days and 7 days post inoculation (dpi).
2. Total RNA was extracted using RNeasy Plant Mini Kit, DNaseI treated and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit. Relative levels of PVY RNA and expression of CAB were followed using qPCR.

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3. Reaction mixtures and sample dilutions were prepared by hand, master mixes and samples were loaded onto a 384-well plate, and the sample preparation was carried out by the PIPETMAX. The plate was processed in the qPCR cycler, and the data analyzed.

4. The standard curve method was used for relative gene expression quantification, and the transcript of each gene was normalized to COX and Elongation factor-1 (EF1) for potato and PVY, respectively. TaqMan chemistry was used for all genes.

### Sample Analysis with qPCR

Samples were analyzed in 10 µL reactions on in a 384-well plate on the Fast Real-Time PCR System (Applied Biosystems). Each sample was analyzed in triplicate at 25-, 125- and 625-fold dilutions. A standard curve was generated for quantification of each gene (5 dilution points ranging from 5 to 3125-fold dilution were done in 5-fold steps (Figure 2), each step in 5 replicates. In PVY only 4 dilution points were used). Two no template controls (NTCs) were used for each gene.

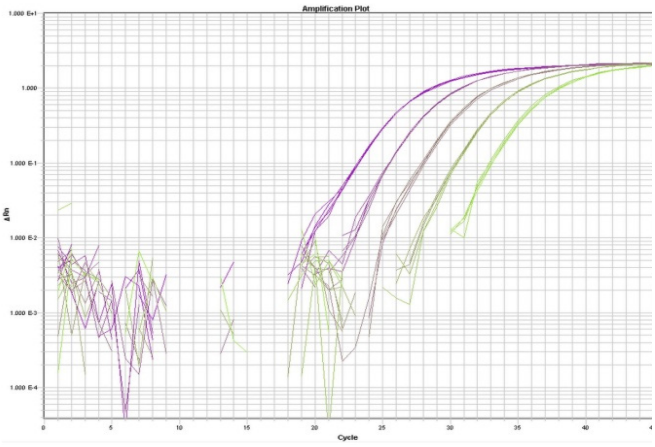


**Figure 2**
COX gene amplification curves of standard 5-fold dilution curve. Amplification curves from the left to right are of the following dilutions: 5x, 25x, 125x, 625x, and 3125x.

## RESULTS AND DISCUSSION

### Gene Expression

The CAB gene is involved in plant photosynthesis, a major primary metabolic pathway in plants. The gene expression of CAB was greater in PVY infected plants than in healthy (mock-inoculated) plant leaves (Figure 3). This showed the higher metabolic rate in infected plants as they were struggling to overcome the viral infection; however, the plants started losing the battle with the virus over the course of several days as CAB gene expression started dropping.

By monitoring the presence of PVY viral RNA in the plants we were able to confirm the presence of the PVY virus in infected plants, and showed that PVY viral RNA accumulated over time. Additionally, the PVY RNA level in 7 dpi plants was higher than in 4 dpi plants, indicating that the virus was spreading and multiplying throughout the plant (Figure 4). No PVY virus was detected in the mock-inoculated plants.

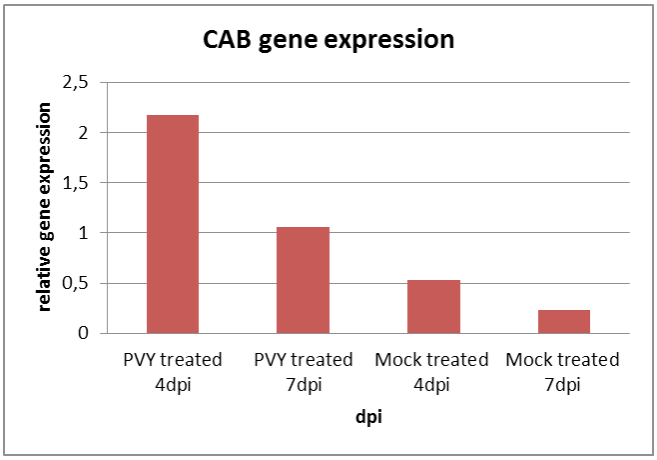


**Figure 3**
Expression of CAB gene normalized to COX in infected and non-infected (mock-inoculated plants).

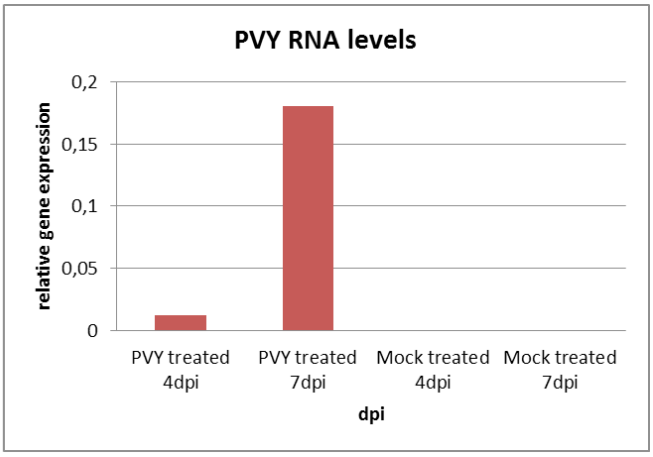


**Figure 4**
Levels of PVY RNA in samples normalized to EF1.

### PIPETMAX® Performance

Performance of PIPETMAX was assessed on three levels: results of negative controls (NTCs), estimations of serial dilutions, and statistical analysis of pipetting.

### Negative Controls

All negative controls (NTCs, no template controls) in the experiment were negative (no signal was detected). This means that no cross-contamination occurred during the qPCR plate pipetting.

### Serial Dilution Estimation

Performing serial dilutions provided an opportunity to assess PIPETMAX's precision. One advantage of serial dilutions was that one can apply linear regression. This also allowed R-squared (Pearson's coefficient of correlation) calculations, which indicated how that individual measurements were close to the trend line (linear regression line in this case).

The optimal value for R-squared is 1.0. Statistical analysis of samples prepared by PIPETMAX reached values exceeding 0.99. (Figure 5).

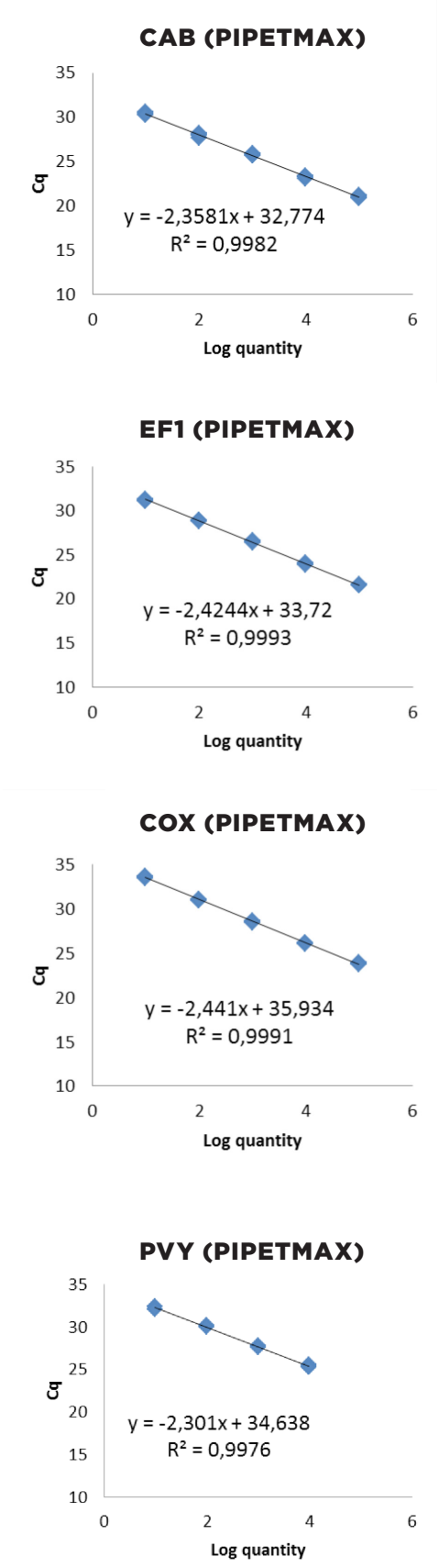


**Figure 5**
Comparison of Pearson's coefficient of correlation calculations across genes.

## Statistical Analysis of Pipetting

Coefficient of variation (CV) is one of the statistical parameters which measures the dispersion of results. In qPCR, CV is usually calculated at the level of copy numbers (final results), and not Cq values.

In Table 1, minimal and maximal CVs for CAB, EF1, and COX genes (across all samples) were calculated. It is worth noting that the variation observed here includes the variation from manual and automatic liquid handling steps.

**Table 1**
Minimal and maximal CVs for CAB, EF1 and COX genes across all samples.

| GENE | CV-PIPETMAX | |
|---|---|---|
| CAB | Min. | 1.9 |
| | Max. | 10.2 |
| | <5% | 40% of samples had CV <5.0% |
| | <15% | 100% of samples had CV <15.0% |
| COX | Min. | 1.3 |
| | Max. | 15.6 |
| | <5% | 67% of samples had CV <5.0% |
| | <15% | 93% of samples had CV <15.0% |
| EF1 | Min. | 1.8 |
| | Max. | 7.9 |
| | <5% | 50% of samples had CV <5,0% |
| | <15% | 100% of samples had CV <15,0% |

## CONCLUSIONS

In this work, CAB expression was followed in the potato cultivar Désirée following PVY infection, and the performance of the PIPETMAX system assessed. After initial infection, CAB expression was increased two-fold at 4 dpi. However, by 7 dpi expression had decreased as the infection progressed. The PIPETMAX system provided a convenient method for preparing qPCR samples, as it did not introduce contamination and eliminated variability.

## REFERENCES

1. Baebler Š, Stare K, Kovač M, Blejec A, Prezelj N, Stare T, Kogovšek P, Pompe-Novak M, Rosahl S, Ravnikar M, Gruden K. 2011. Dynamics of responses in compatible potato-Potato virus Y interaction are modulated by salicylic acid. *Journal of PLoS One.* 6(12): e29009, doi10.1371-0029009.

2. Bilgin DD, Zavala JA, Zhu J, Clough SJ, Ort DR, DeLucia EH. 2010. Biotic stress globally downregulates photosynthesis genes. Journal of *Plant, cell & environment.* 33(10): 1597–613, doi10.1111, j1365-3040.2010.02167.