



Precise Evaluation of Plant RNA Extraction Methods with Automated RT-qPCR Assay Preparations

Application Note TRANS0414



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Introduction

Determining the quantity and quality of DNA or RNA after different extraction techniques can be difficult because most analysis methods alone are insufficient. Spectrophotometry is commonly used to quantitate nucleic acid, however, this approach can be misleading because contaminants, such as dsDNA, RNA, oligonucleotides, and free nucleotides can contribute to the overall A260 readings, skewing the results. Fluorescent dye binding nucleic acid detection methods are very sensitive and somewhat selective, but information on sample purity and integrity is not provided. Real time-qPCR, alternatively, is highly sensitive, has a wide dynamic range, and can provide some insight into the quality of the extracted DNA or RNA for downstream applications.

The amount of amplifiable nucleic acid is dependent on multiple factors including the degree of degradation, co-purification of contaminants that may inhibit enzymatic assays, and DNA integrity in cross linking experiments (common in FFPE- preserved samples). Amplification conditions will also influence success (e.g. amplicon size, reagents and primer design). Promega GoTaq® amplification reagents, such as the GoTaq® Probe 1-Step RT-qPCR system, were specifically designed to be highly robust and resistant to enzyme inhibitors, improving the quality of the results.

In this study, functional corn RNA was quantitated by RT-qPCR using the GoTaq® Probe 1-Step RT-qPCR System after two different extraction methods: Promega's Maxwell® 16 LEV (low elution volume) Plant RNA Kit and a competitor spin column-based plant RNA extraction kit. To enhance the pipetting accuracy and consistency an automated pipetting assistant, Gilson PIPETMAX® 268, was used to reduce pipetting errors inherent in manual methods (Figure 1).

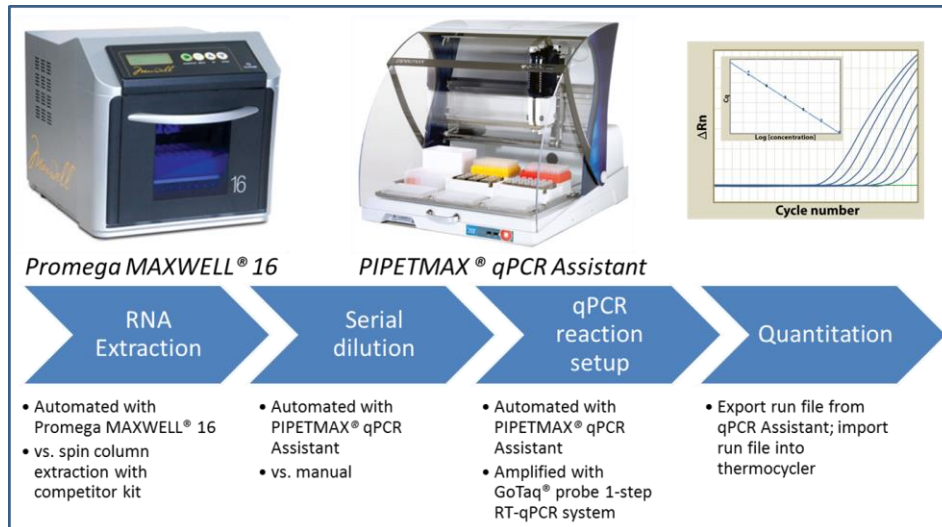


Figure 1. Experimental workflow.

Materials & Methods

RNA Extraction

Corn (*Zea mays*) leaf samples were ground in liquid nitrogen using a mortar and pestle. 60mg of ground leaf tissue were used in all isolations. Automated isolation of RNA was carried out according to manufacturer's instructions using the Maxwell® 16 LEV Plant RNA Kit (Promega, Cat. #AS1430). RNA purifications were performed in replicates of 6 following the manufacturers' recommendations (Promega Technical manual – TM415). Spin column RNA isolation was carried out according to manufacturer's instructions using a competitor's plant RNA kit.

Quantitative RT-PCR

Corn Total RNA (Zyagen, Cat. #PLR-1002) was used as a positive control. TaqMan® Corn Primers and Probe [Cat. #Zm04073989_m1 (zmm19)] were obtained from Life Technologies. The PIPETMAX qPCR Assistant software (Gilson) creates 3 separate protocols for the reaction preparation (master mix preparation,

sample/standard dilution, and qPCR plate protocol); all three steps were completed on PIPETMAX. For greatest consistency, master mixes prepared by PIPETMAX were mixed manually after assembly. The standard curve was amplified in triplicate, using the control Corn Total RNA diluted in 4-fold steps from 500ng/μl to 0.12ng/μl. Samples were amplified in replicates of six (n=6) at a 1:10 dilution (dilutions were completed on the PIPETMAX) to bring the concentrations within the standard curve range. For comparison to manual amplifications, the standard and samples (neat and 1:10 dilution) were amplified in duplicate. The RNA eluates were amplified on a Bio-Rad CFX96 Real-Time System using the GoTaq® Probe 1-Step RT-qPCR System (Promega, Cat. #A6120) according to manufacturer's recommendations (Promega Technical manual – TM379). All reactions were prepared with fixed volume (2μl) sample additions in a final volume of 20 uL.



Results and Discussion

Corn RNA was isolated from a single source of liquid nitrogen-ground leaf tissue using two different methods: the semi-automated Maxwell® 16 system and a competitor manual spin column-based method. To determine the amount of amplifiable RNA, the eluates were reverse transcribed and amplified using the GoTaq® probe 1-step RT-qPCR system. The PIPETMAX was used to prepare sample dilutions, control corn RNA standard serial dilutions, and amplification reactions. The control standard curve yielded a linear fit analysis with an R^2 value of 0.9989, with minimal variation between replicates (Figure 2).

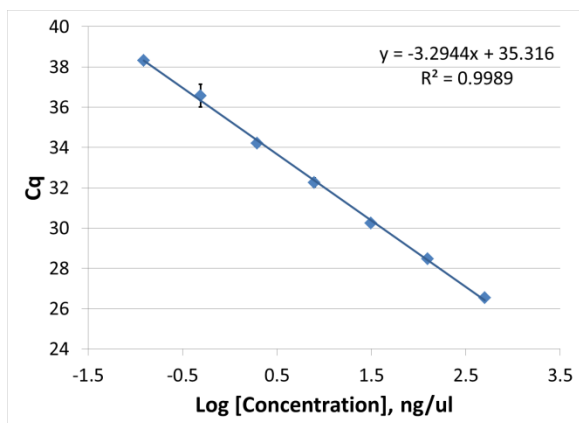


Figure 2. Control corn standard curve used to calculate the amount of amplifiable RNA in sample purifications. All amplification reactions were prepared on the PIPETMAX. Cq values are shown as AVG \pm 1SD for $n=3$.

All amplification reactions resulted in Cq values that fit within the standard curve, allowing for the determination of all eluate concentrations. The calculated RNA concentrations were similar between the two isolation methods (Figure 3). The Maxwell® 16 System is just as efficient at isolating amplifiable RNA as the competitor spin method while also enabling a walkaway sample

purification method. The coefficient of variation (%CV) values which indicates the extent of variability in relation to mean of the population were calculated and resulted in values $<10\%$ per sample, with an average %CV of 6.9 for Maxwell® 16 eluates and 6.4 for the competitor spin method. This indicates low variation in the replicate samples.

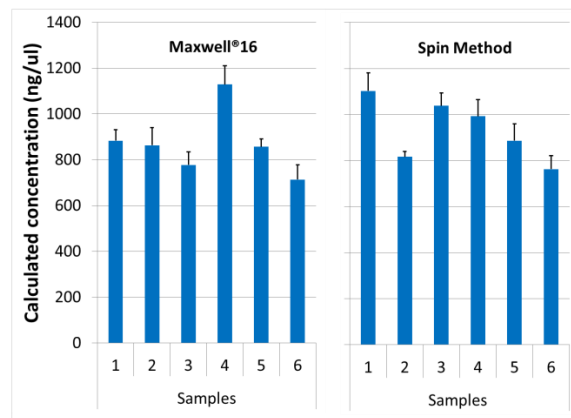


Figure 3. Calculated concentrations of amplifiable RNA in sample isolations. RNA was purified from replicate samples using either the Maxwell®16 or a competitor spin method. All amplification reactions were prepared in sextuplet on the PIPETMAX. Concentrations are shown as AVG \pm 1SD ($n=6$).

To evaluate the PIPETMAX pipetting accuracy and variability versus manual pipetting, we tested the linear fit of a serial diluted standard and variability of sample quantitation when amplified using RT-qPCR. A standard curve was made by serially diluting the control corn RNA both manually and on the PIPETMAX. Both the manual and PIPETMAX standard curves were very good with R^2 values of 0.996 and 0.995 respectively (Figure 4). The manual standard curve did show more variation on the last dilution point and had a slightly lower slope (-3.17 to -3.21 respectively).

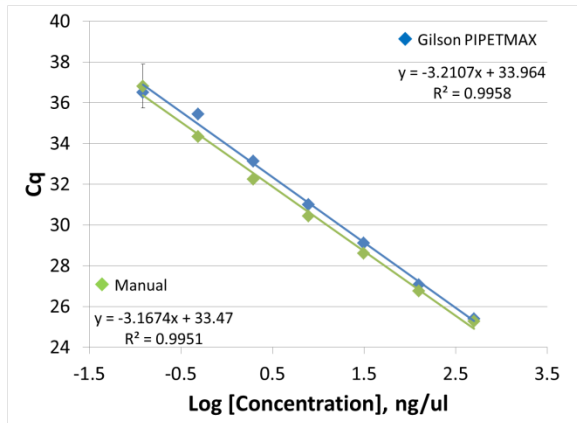


Figure 4. Control corn standard curves prepared manually as well as on the PIPETMAX. Cq values are shown as AVG ± 1SD (n=2).

Additional RNA samples were quantitated using qPCR reactions prepared on the PIPETMAX qPCR Assistant or manually. The calculated RNA concentrations between the PIPETMAX and manual amplifications were comparable; however, the manually pipetted samples were consistently more variable than the ones prepared on the PIPETMAX (Figure 5).

An analysis of the %CV values from these data show that the PIPETMAX had lower values as compared to the manual preparation (Table 1). This indicates that the PIPETMAX can pipet reagents with less error than a scientist pipetting manually.

Summary

- The Promega Maxwell® 16 instrument provides consistent RNA purifications from plant samples with minimal preprocessing.
- The instrument enables walkaway purification, allowing greater productivity in other areas of the lab.
- The RNA purifications from the Maxwell® 16 had similar yields and quality as the more time-intensive and laborious competitor spin method.
- Integration of the PIPETMAX with the GoTaq™ Probe 1-Step RT-qPCR System provided a convenient and consistent method of automating the preparation and execution of the amplification-based quantitation assay.
- Further testing showed the PIPETMAX qPCR Assistant is a good substitute for manual preparation of amplification reactions and can help reduce variability introduced through manual pipetting.

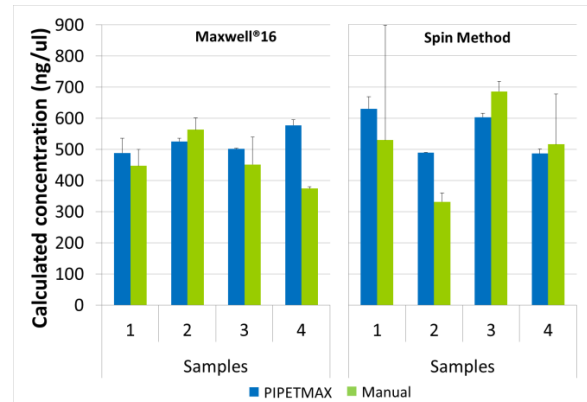


Figure 5. Calculated concentrations of amplifiable RNA in sample isolations. RNA was purified using the Maxwell®16 or a competitor spin method. Amplification reactions were prepared using PIPETMAX or manually. Data are shown as AVG ± 1SD (n=2).

Table 1. Average %CV from the sample amplifications (duplicate amplifications of quadruplicate purifications). Purifications were carried out using the Maxwell® 16.

Method	Dilution	Average %CV
PIPETMAX	Neat	3.3
	1/10	7.1
Manual	Neat	19.0
	1/10	9.0