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

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Abstract # 2050

Overview

Obtaining high-quality RNA from biological samples is the first and most important step in performing many molecular biology techniques including RNA sequencing (RNA-seq), quantitative reverse transcription PCR (RT-qPCR), RNA interference (RNAi), and cDNA cloning. The effectiveness of RNA extraction techniques is often based on the yield and purity level of the prepared sample, but common methods for determining these parameters, such as spectrophotometry and fluorescent dye-binding, alone are limited and inconclusive. Here we apply real time-qPCR as an alternative technique to quantitate extracted RNA (cDNA) from corn leaves (as an example) to assess the integrity and functionality of the nucleic acid for downstream applications. Two RNA extraction techniques were tested: manual solid-phase extraction utilizing a spin column kit and magnetic bead–based separation utilizing the Promega Maxwell® 16 LEV Plant RNA kit. The quality and quantity of amplifiable RNA was then determined by RT-qPCR using Promega's GoTaq® Probe 1-step RT-qPCR assay. To further enhance the accuracy and consistency of the procedures, Gilson's PIPETMAX® qPCR Assistant was employed to automate the qPCR process. The Maxwell® 16 LEV Plant RNA extraction system produced similar yields of corn RNA as the traditional spin column method, while affording a walkaway work solution. Integration of the PIPETMAX® qPCR Assistant with the GoTaq® Probe 1-step RT-qPCR assay reduced the RT-qPCR data variability more than two-fold over manual pipetting methods. Preparation of consistently high quality RNA using automated RNA extraction and RT-qPCR assay systems expedites downstream molecular biology applications leading to quicker and more reliable results.

Methods

Experimental Workflow (Figure 1)

- RNA Extraction: RNA was extracted from processed corn
- qPCR reaction setup: Master mixes, standards and sample dilutions, and qPCR mixes were created both manually and with the PIPETMAX® qPCR Assistant
- RT-qPCR: Thermocycling and detection were performed using the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System with CFX Manager™ Software

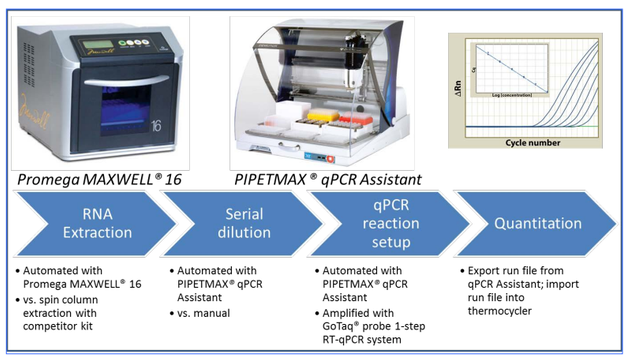

Figure 1. *Experimental Workflow*

RNA Extraction & Isolation

- Corn (*Zea mays*) leaf sample was ground in liquid nitrogen using a mortar and pestle
- 60mg (each) of ground leaf tissue were used in all isolations
- RNA purifications were performed in replicates of six
- Semi-automated isolation of RNA: Maxwell® 16 LEV Plant RNA Kit (Promega, Cat. #AS1430)
- Spin column RNA isolation: competitor's plant RNA kit

RT-qPCR

- RNA eluates were reverse transcribed and amplified using the GoTaq® Probe 1-Step RT-qPCR System (Promega, Cat. #A6120) TaqMan®
- Corn Primers and Probe [Life Technologies, Cat. #Zm04073989_m1 (zmm19)]
- Corn Total RNA (Zyagen, Cat. #PLR-1002) was used as a positive control
- 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
- Reactions prepared with fixed volume (2µl) sample additions in a final volume of 20µl
- The PIPETMAX® qPCR Assistant generates the run file specific for the Bio-Rad CFX96 for simplified sample tracking
- To compare the reliability and accuracy of the PIPETMAX® qPCR Assistant, the standards and samples (neat and 1:10 dilution) were also processed manually and in duplicate

Results

RNA Extraction technique

- qPCR data was analyzed using Bio-Rad software
- The control standard curve yielded a linear fit analysis with an R^2 value of 0.9989 (Figure 2)
- 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 coefficient of variation (%CV) values were <10% per sample
  - Maxwell® 16 = 6.9 %CV
  - Competitor spin method = 6.4 %CV

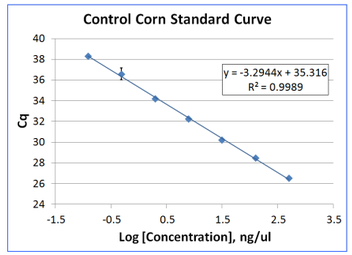

Figure 2. *Control corn standard curve used to calculate the amount of amplifiable RNA in sample purifications. All amplification reactions were prepared on the PIPETMAX® qPCR Assistant. Cq values are shown as AVG ± 1SD for n=3.*

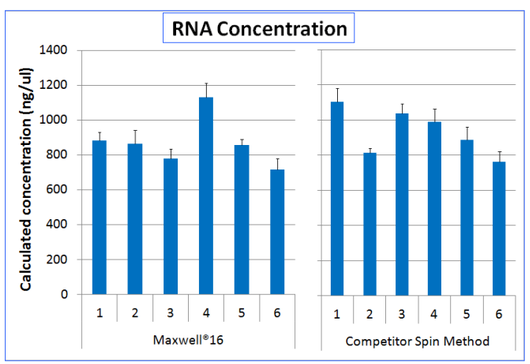

Figure 3. *Calculated concentrations of amplifiable RNA in sample isolations. RNA was purified from replicate samples using either the Maxwell®16 or a competitor spin method. All amplification reactions were prepared in sextuplet on the PIPETMAX® qPCR Assistant. Concentrations are shown as AVG ± 1SD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

PIPETMAX® qPCR Assistant accuracy

- Standard curve R^2 values (Figure 4)
  - Manual = 0.996
  - PIPETMAX® qPCR Assistant = 0.995
- Slope (Ideal = -3.3)
  - Manual = -3.17
  - PIPETMAX® qPCR Assistant = -3.21
- Calculated RNA concentrations between the PIPETMAX® qPCR Assistant and manual amplifications were comparable (Figure 5)
- Manually pipetted samples were consistently more variable than the ones prepared on the PIPETMAX® qPCR Assistant as shown by a comparison of %CV (Table 1)

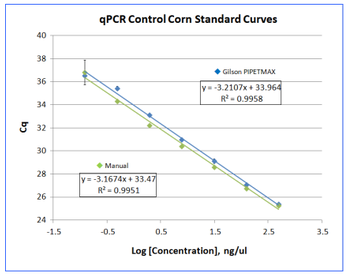

Figure 4. *Control corn standard curves prepared manually as well as on the PIPETMAX® qPCR Assistant. Cq values are shown as AVG ± 1SD (n=2).*

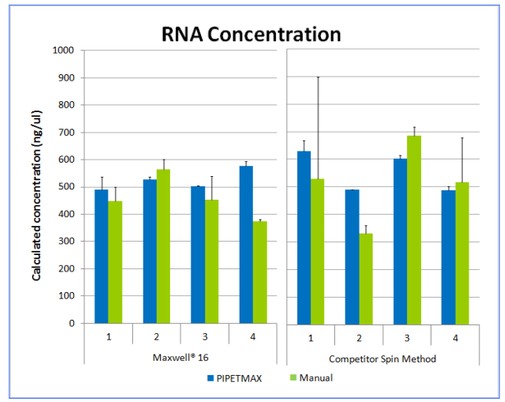

Figure 5. *Calculated concentrations of amplifiable RNA in sample isolations. RNA was purified using the Maxwell®16 or a competitor spin method. Amplification reactions were prepared using PIPETMAX® qPCR Assistant or manually. Data are shown as AVG ± 1SD (n=2).*

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® qPCR Assistant	Neat	3.3
	1/10	7.1
Manual	Neat	19.0
	1/10	9.0

Conclusions

- 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® qPCR Assistant 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.

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