

Non-Organic-Based Isolation of Plant microRNA using Norgen's Plant/Fungi RNA Purification Kit

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INTRODUCTION

Small RNAs, including microRNAs (miRNAs) and short interfering RNAs (siRNAs), are key components of an evolutionarily conserved system of RNA-based gene regulation in eukaryotes. In eukaryotes, regulatory small RNAs are divided into two main classes; (a) small interfering RNAs (siRNAs) are double-stranded RNA of ~20-25 nucleotides that are involved in RNA interference, and (b) microRNAs (miRNAs) are single-stranded RNA of ~21-23 nucleotides in length that contain complementary sequences to the 3' untranslated regions of the target messenger RNAs (mRNA). They are involved in many molecular interactions, including defense against viruses and regulation of gene expression during development. miRNAs interfere with expression of messenger RNAs encoding factors that control developmental timing, stem cell maintenance, and other developmental and physiological processes in plants and animals. miRNAs are negative regulators that function as specificity determinants, or guides, within complexes that inhibit protein synthesis (animals) or promote degradation (plants) of mRNA targets (1).

Unlike larger DNA or RNA molecules, small RNAs are subjected to significant loss in traditional isolation methods that involve alcohol precipitation. Moreover, some available commercial products for small RNA isolation involve the use of organic extraction, which is hazardous and time-consuming. Norgen's Plant/Fungi RNA Purification Kit provides an innovative and rapid method for the isolation and purification of total RNA, including small RNA, from both plant and fungal cells that does not require organic extraction. The procedure is based on spin column chromatography using Norgen's proprietary resin as the separation matrix. The procedure is rapid and convenient, as it does not rely on the use of liquid nitrogen in order to homogenize the samples. The purified RNA is of the highest quality and can be used in a number of downstream applications including real time PCR, reverse transcription PCR, Northern blotting, and expression array analysis.

In this application note, Norgen's Plant/Fungi RNA Purification Kit is used to isolate total RNA from a variety of different plant cells. The yield and integrity of the purified

RNA is then analyzed by gel electrophoresis, as well as by using an Agilent BioAnalyzer. Furthermore, the downstream application of RT-PCR is performed, indicating the purity and biological activity of the purified RNA.

METHODS AND MATERIALS

Plant RNA Isolation

Plant RNA was isolated from 50 mg of plant leaf tissue (equivalent to ~ 5 x 10⁶ plant cells) using Norgen's Plant/Fungi RNA Purification kit as per the provided protocol (Figure 1). Briefly, the plant cells were ground in a mortar containing 600 µL of Lysis Solution with a pestle until the tissue was completely macerated. The lysate was then transferred into an RNase-free microcentrifuge tube and centrifuged for 2 minutes to remove cellular debris. The supernatant was then transferred to a new RNase-free microcentrifuge tube and an equal volume of 70% ethanol was added and mixed by vortexing. Next, 600 µL of the clarified lysate was then loaded onto an assembled column and centrifuged for 1 minute at 14,000 x g (~14,000 rpm). The flow-through was discarded and the column reassembled. The remaining lysate was then loaded onto the same column by centrifugation for 1 minute at 14,000 x g. The column was then washed a total of three times by applying 400 µL of Wash Solution to the column, centrifuging for 1 minute and then discarding the flow-through. The columns were centrifuged for 2 minutes to thoroughly dry the resin. For RNA elution the column was placed into a fresh 1.7 mL elution tube and 50 µL of the Elution Buffer was applied to the column. Columns were then centrifuged for 2 minutes at 200 x g (~2000 rpm), followed by a 1 minute spin at 14,000 x g. Purified RNA was then stored at -20°C for several days or at -70°C for long term storage.

For comparison of microRNA isolation, total RNA was also isolated from the same plant samples using a leading market competitor's plant RNA purification kit according to the manufacturer's protocol and used in comparative experiments.

RNA Gel Electrophoresis

The purified total plant RNA (Norgen's and competitor's) was run on 1X MOPS, 1.0% formaldehyde-agarose gels for visual inspection and comparison. Generally, 5 µL of each 50 µL elution was run on the gel. The purified RNAs (Norgen's and competitor's) were also resolved on an 8% Urea-PAGE gel for visual comparison.

Capillary Electrophoresis

Purified RNAs from grape, tomato, tobacco and peach leaf tissue were loaded onto an Agilent® RNA Nano 6000 chip and resolved on an Agilent® 2100 BioAnalyzer according to the manufacturer's instructions.

RT-qPCR Assay

Plant RNA purified from peach leaves was used as template for one step RT-qPCR, and miRNA was detected by employing primers specific to miR398b.

Macerate cells or tissue in a mortar using **Lysis Solution**

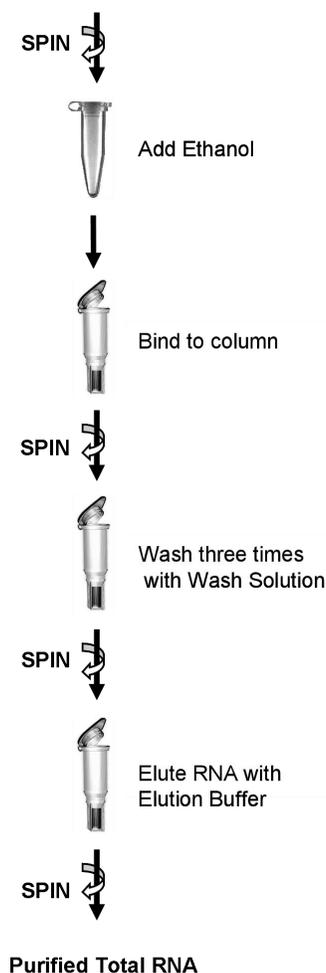


Figure 1. Procedure Flowchart for the Purification of Total RNA from Plant Cells using Norgen's Plant/Fungi RNA Purification Kit.

RESULTS AND DISCUSSION

Total RNA was isolated from 5×10^6 plant cells using Norgen's Plant/Fungi RNA Purification Kit according to the provided protocol as described in Figure 1. The entire protocol was completed in 30 minutes. At the same time, a

commercially available competitor's plant total RNA kit was used for comparison. Once total RNA was isolated from the leaf tissues of apple, peach, grape, strawberry and pine needles, they were run on a 1X MOPS, 1.0 % formaldehyde-agarose gel for visual inspection (Figure 2). As it can be seen in Figure 2, RNA samples prepared using Norgen's Plant/Fungi RNA Purification Kit were of a high quality. In addition, Norgen's kit allowed for the isolation of RNA from all sample types, whereas the competitor's kit was not successful at isolating RNA from some of the more difficult sample types including grape and strawberry leaves and pine needles. Importantly, microRNA and small RNAs could be observed in the total RNA isolated using Norgen's kit. In contrast, the competitor's total RNA kit did not isolate small RNAs. Thus Norgen's Plant/Fungi RNA Purification Kit truly isolates total RNA with a wider size diversity and quality than its competitor's, including RNA from difficult samples.

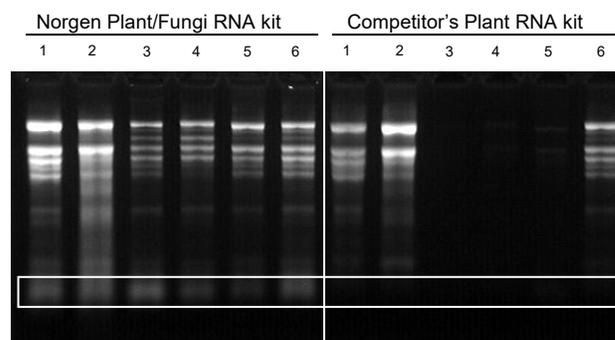


Figure 2. Isolation of True Total RNA, Including Small RNA Species, Using Norgen's Kit.

Total RNA was isolated from 50 mg ($\sim 5.0 \times 10^6$ cells) of Apple (1), Peach (2), Grape (3), Pine needle (4), Strawberry (5) or Pear (6) leaf tissue using Norgen's Plant/Fungi RNA Purification Kit and a leading competitor's kit. Samples of the purified RNA (5 μ L of each 50 μ L elution) were loaded onto a 1X MOPS, 1.0% formaldehyde-agarose gel and visualized via ethidium bromide staining. Norgen's kit allowed for the isolation of high quality RNA from all of the samples, including the difficult samples, while the competitor failed to isolate RNA in some cases. Furthermore, only Norgen's kit was able to isolate the small RNA species (white box).

The quality of small RNAs isolated by Norgen's purification kit was further demonstrated by resolution on an 8% Urea-PAGE gel (Figure 3) and capillary gel electrophoresis (Figure 4). Total RNA was isolated in duplicate from apple and peach leaf tissue using Norgen's purification kit and the competitor's kit. Figure 3 demonstrates that unlike its competitor, Norgen's kit is able to isolate small RNA species which are < 200 nucleotides in length.

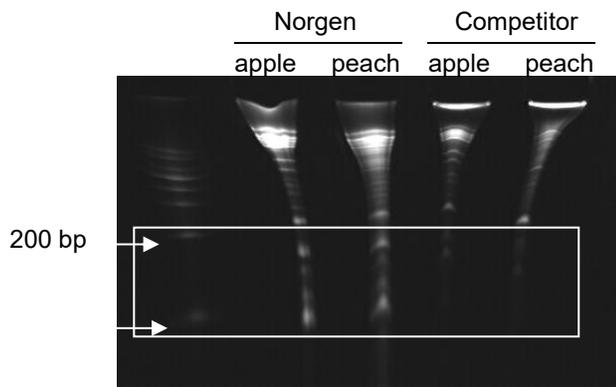


Figure 3. Resolution of Small RNA on an 8% Urea-PAGE Gel. Following purification of total RNA from apple and peach leaf tissue using Norgen's Plant/Fungi RNA Purification Kit and a competitor's kit, the RNA was separated on an 8% urea-PAGE gel (7 μ L loaded from the 50 μ L elutions). Norgen's kit was able to isolate true total RNA with a wide size diversity, including small RNA species (white box). In contrast, the competitor's total kit was unable to purify RNA species which were below 200 nucleotides in size.

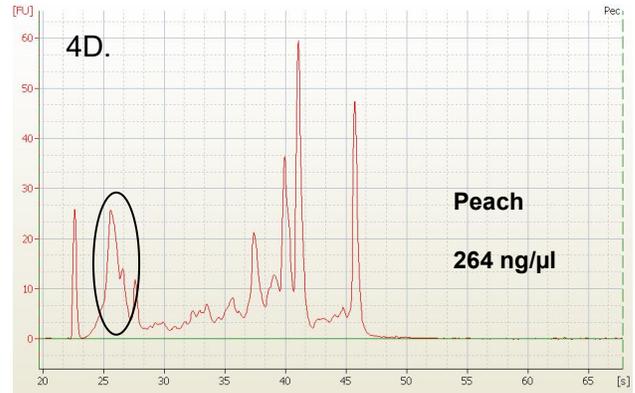
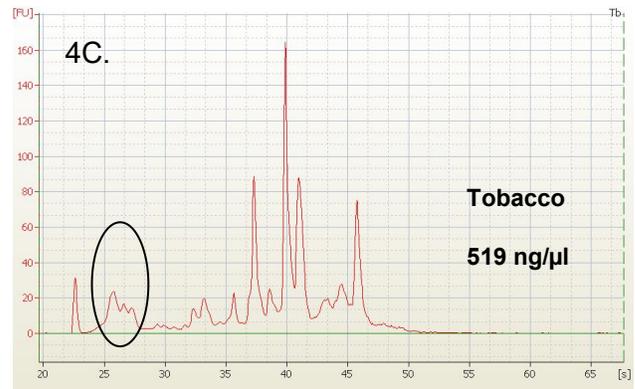
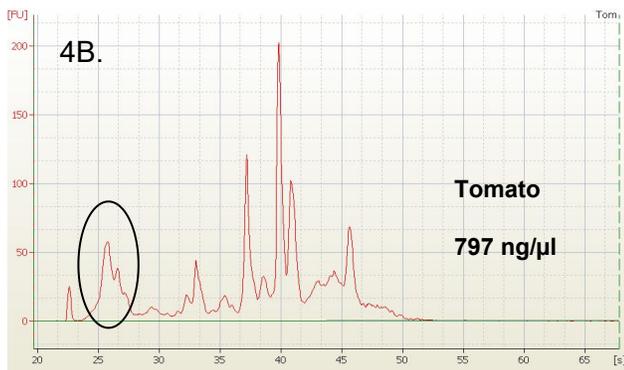
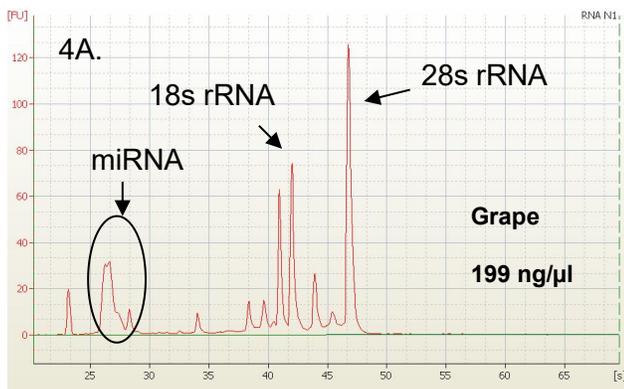


Figure 4. Resolution of Total RNA Isolated Using Norgen's Kit on the Agilent BioAnalyzer.

Total RNA was isolated from grape (Panel A), tomato (Panel B), tobacco (Panel C) and peach (Panel D) leaf tissue using Norgen's kit. Purified DNA was then resolved on an Agilent Lab-on-a-Chip and electropherograms were generated. All RNA species, including small RNA species, can be detected in all 4 cases.



The ability of Norgen's Plant/Fungi RNA Purification Kit to isolate true total RNA, including small RNA, was further demonstrated when total RNA samples purified from grape, peach, tomato and tobacco were resolved on an Agilent Lab-on-a-Chip (Figure 4). Panel A in Figure 4 is an electropherogram of total RNA isolated from grape leaf tissue using Norgen's purification kit. All the RNA species, including microRNA, 18s rRNA and 28s rRNA can be observed. Similar results were obtained when total RNA was purified from tomato (Panel B), tobacco (Panel C) or peach (Panel D). It is evident that RNA species of a wide size diversity can be purified using Norgen's Plant/Fungi RNA Purification Kit, including plant microRNAs.

In order to analyze the biological activity of the purified RNAs, RT-qPCR was performed. Unlike regular RT-PCR, the amplification and detection of small RNA molecules, such as microRNA, requires the addition of an adaptor. One of the commonly-used protocols involves the addition of a poly(A) tail to the microRNA by Poly(A) Polymerase (2). This method was employed here, and Figure 5 shows the amplification of the miR398b transcript from total plant RNA isolated from peach leaves using Norgen's Plant/Fungi RNA Purification Kit. The PCR product was successfully detected from the total RNA purified from peach leaf tissue.

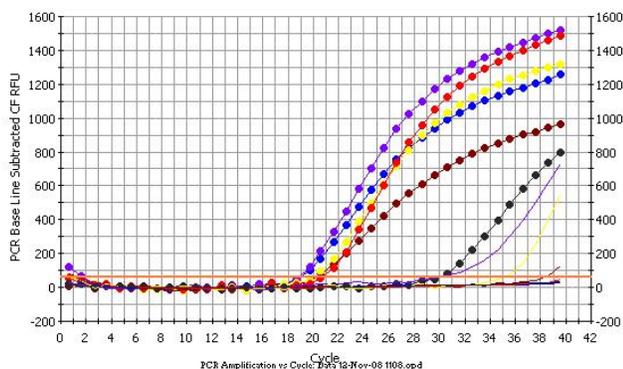


Figure 5. One-step RT-qPCR for the Detection of Plant miRNA.

Total RNA was extracted from peach leaf tissues using Norgen's Plant/Fungi RNA Purification Kit, and miRNA was detected using primers specific for miR398b. miRNA was detected for all stem loop primer concentrations tested: 5nM (red); 10nM (yellow); 25nM (purple); 50nM (blue) and; 100nM (burgundy).

CONCLUSION

Through the analysis of the performance of Norgen's Plant/Fungi RNA Purification Kit for isolating total RNA from plant cells, a number of conclusions regarding Norgen's kit can be made:

1. Norgen's kit allows for the isolation of high quality total RNA, including small RNA, within 30 minutes and without the use of any organic solvents. Unlike other commercial kits, Norgen's kit does not require the use of organics for extraction, or the use of liquid nitrogen for homogenization of samples, making the RNA purification rapid and convenient.

2. Norgen's kit isolates RNAs of high yield, purity and integrity from a wide range of plant and fungal samples. The purity and integrity of the total RNA isolated using Norgen's kit could be seen in the various gel photos. In addition, the RNA was of a high quality, as it could be used in downstream applications including RT-qPCR.

3. Norgen's kit isolates true total RNA, including small RNA, from various plant samples. As can be seen in the various figures, Norgen's kit was able to consistently isolate total RNA, including microRNA. The competitor's kit failed to isolate the small RNA species. Furthermore, Norgen's kit was able to isolate RNA from all the samples tested, while the competitor failed to isolate RNA from some of the more difficult samples.

REFERENCES

1. Carrington, J. C. and V. Ambros. 2003. Role of microRNAs in Plant and Animal Development. *Science*. 301: 336.
2. Shi, R. and V. L. Chang. 2005. Facile means for quantifying microRNA expression by real-time PCR. *BioTechniques*. 39: 519-24.

