Non-Organic-Based Isolation of Mammalian microRNA using Norgen's microRNA Purification Kit

B. Lam, PhD[1], P. Roberts, MSc[1] Y. Haj-Ahmad, M.Sc., Ph.D[1,2]
[1]Norgen Biotek Corporation, Thorold, Ontario, Canada
[2]Centre for Biotechnology. Brock University, St. Catharines, Ontario, Canada

INTRODUCTION

Small RNAs that are non-protein-coding have attracted much attentions in recent years for their role in regulating gene expression in signaling pathways, cell death, organ development and metabolism (1,2). Moreover, increasing evidence has suggested the involvement of small RNAs in human disease including cancer pathogenesis and viral-related infections (3). 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). Two types of small RNAs are also found in prokaryotes such as *E coli* (4); (a) RNA-binding small RNAs that act by basepairing with target mRNA, and (b) protein-binding small RNAs that interact with translational regulator proteins.

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 involve the use of organic extraction which is hazardous and time-consuming. Therefore, there is a great need for a simple and convenient method for the isolation and purification of small RNAs.

Norgen's microRNA Purification Kit provides an innovative and rapid method for the isolation and purification of microRNA and small RNA from cultured animal cells, tissues, bacteria and plants. The procedure is based on spin column chromatography, using Norgen's proprietary resin as the separation matrix. The purified RNA 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 microRNA Purification Kit is used to isolate microRNA from HeLa cells. Furthermore, the downstream application of RT-PCR is performed, indicating the purity and biological activity of the purified RNA.

METHODS AND MATERIALS

microRNA Isolation

microRNAs were isolated from 5 x 10^5 HeLa cells using Norgen's microRNA Purification Kit as per the provided protocol (Fig. 1). Briefly, the cells were pelleted by centrifugation, the media removed, and the pellet washed once with PBS. Lysis solution was then added to the cell pellet, followed by the addition of ethanol. The lysate was then loaded onto a provided Large RNA Removal Column and the flowthrough, containing the microRNAs, was collected. Additional ethanol was added to the flowthrough, and then the lysate-ethanol mix was loaded onto a provided microRNA Enrichment Column. The column was then washed three times with 400 µL of the Wash Solution. The purified microRNAs were eluted with 50 µL (or as little as 20 µL) of Elution Solution. The large RNA that was bound to the Large RNA Removal Column was also eluted using 50 µL of the Elution Solution. microRNA isolation using a competitor's kit was also performed in parallel using the same amount of HeLa cell input.

RNA Gel Electrophoresis

The purified RNAs (from both Large RNA Removal Column and microRNA Enrichment Column) were run on 1.8% formaldehyde-agarose gels for visual inspection of large RNA removal in the microRNA fraction. Generally, 10 µL of each 50 µL elution was run on the gel. The purified microRNAs (Norgen's and competitor's) were also resolved on 8% Urea-PAGE gel for visual comparison.

3430 Schmon Parkway
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Capillary Electrophoresis

The purified RNAs were loaded onto an Agilent® RNA Nano 6000 chip and resolved on an Agilent® 2100 BioAnalyzer according to manufacturer's instruction. Total HeLa cells RNA isolated using Norgen's Total RNA Isolation Kit was used as a control.

RT-PCR Assay

microRNAs purified from HeLa cells were modified according to (5) for RT-PCR. Briefly, the purified microRNAs were polyadenylated by Poly(A) Polymerase at 37°C for 1 hour. The tailed RNAs were then purified using Norgen's RNA Cleanup and Concentration Kit as per the provided protocol. First-strand cDNA synthesis was performed using Invitrogen's Superscript II system and a poly(T) adaptor primer (2). The cDNAs were then used as the template in PCR reactions. For miRNA amplification, primers specific for the human *miR-21* (5′ CGTGACGTTAGCTTATCAGACTG 3′) and the adaptor (according to (5)) were used. Also, 5S rRNA (Forward Primer 5′ GCCATACCACCCTGAACG 3′; Reverse Primer 5′ AGCCTACAGCACCCGGTATT 3′) amplification was used as internal control.

RESULTS AND DISCUSSION

microRNAs was isolated in duplicate from 5 x 10^5 HeLa cells using Norgen's microRNA Purification Kit as per the provided protocol (Figure 1). The entire protocol was completed in 20 minutes. A commercially available competitor's microRNA isolation kit was used in parallel for comparison. In contrast to Norgen's kit, the time needed to complete the protocol was over 40 minutes. Once the RNAs were isolated, they were run on Urea-PAGE gel for visual inspection.

The Urea-PAGE gel in Figure 2 demonstrated that the small RNAs purified by Norgen's protocol were of a high quality, with no signs of degradation of the major rRNA or tRNA species. More importantly, Norgen's kit truly isolated only RNAs of size <200 nucleotides (Fig. 2, Lanes 3 and 4). Only the three major tRNA bands are present on the gel, with no other contaminating bands. In contrast, RNA isolated by the competitor's kit contained some RNA species that were over 200 nucleotides in size (Fig. 2, Lanes 1 and 2). This is of particular significance as most small regulatory RNAs are in the size range of 15 to 30 nucleotides.

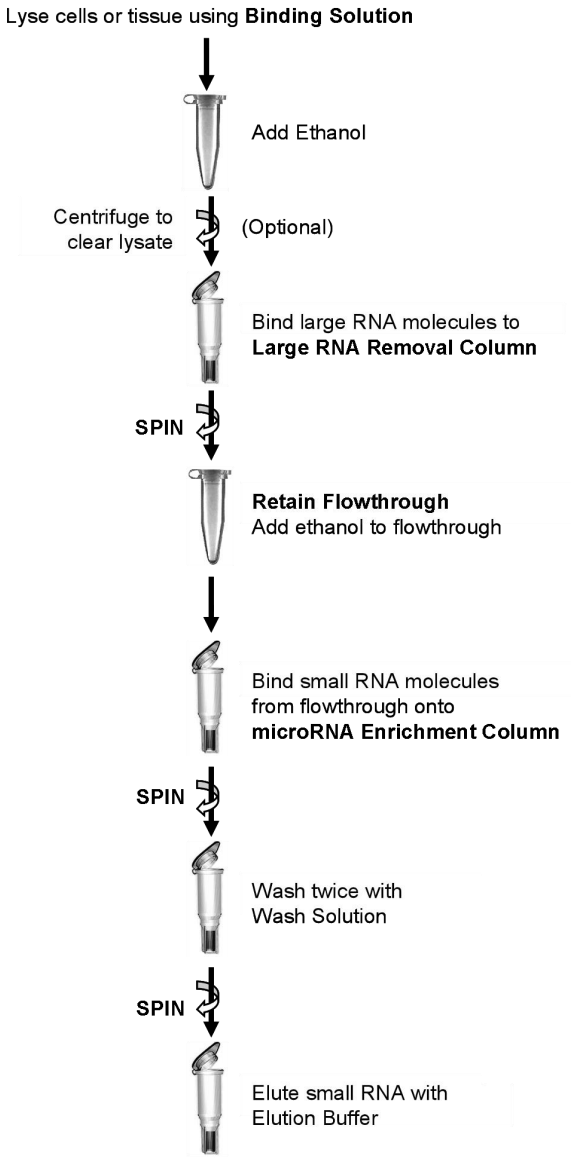

Figure 1. Procedure flowchart for the purification of microRNA from HeLa cells using Norgen's microRNA Purification Kit.

Thus, Norgen's kit offers a quick, non-organic-based method to isolate high quality small RNAs that are truly less than 200 nucleotides in size.

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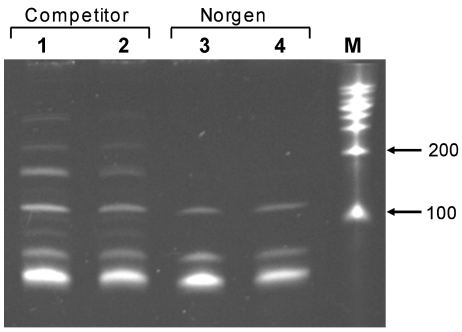

Figure 2. Isolate True microRNA using Norgen's Kit
microRNA was isolated from 5 x 10^5 HeLa cells using Norgen's microRNA Purification Kit (Lanes 3 and 4) and a competitor's kit (Lanes 1 and 2). Samples of the purified small RNA were run on an 8% urea-PAGE gel. Lane M is Norgen's 100b RNA Ladder.

The quality of small RNAs isolated by Norgen's microRNA Purification Kit was further demonstrated by agarose-gel electrophoresis and capillary gel electrophoresis (Figure 3). Figure 3 shows that in formaldehyde-agarose gel electrophoresis, the eluted small RNAs (Lanes 3 and 4) are free of any large RNAs including the major 28S and 18S rRNA (Lane 1 and 2). Similar results were obtained when the RNAs were resolved on an Agilent® Lab-On-A-Chip (Figure 4). Panel A in Figure 4 is an electropherogram of total RNA isolated from HeLa cells using Norgen's Total RNA Purification Kit. All the RNA species, including microRNA, 18S rRNA and 28S rRNA can be see. Panel B in Figure 4 shows the 28S and 18S rRNA that is removed using the Large RNA Removal Columns, while Panel C in Figure 4 shows the microRNA that is isolated using Norgen's microRNA Purification Kit. Clearly, no microRNA is being lost during the large RNA removal (Panel B), and all the large RNA species have been completely removed and only the microRNA is being recovered in the final elution (Panel C).

Furthermore, the results in Figure 4 Panel C show the compatibility of the small RNAs isolated with Norgen's microRNA Purification Kit with a Bioanalyzer. This provides added benefits for quantifying the isolated microRNA, as spectrophotometry may not be applicable for quantifying the small RNA, particularly when low input amounts are used.

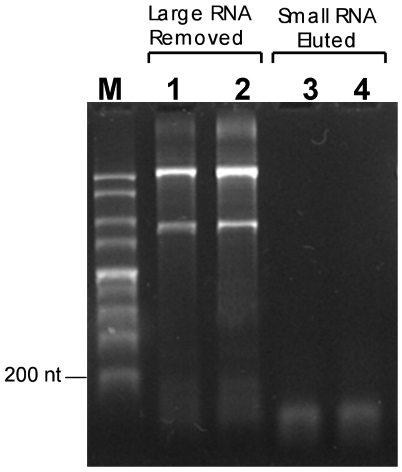

Figure 3. Efficient Removal of Large RNA Species. Norgen's microRNA Purification Kit was used to isolate small RNA species from HeLa cells. The RNA was eluted from both the Large RNA Removal Column (Lanes 1 and 2) and the microRNA Enrichment Column (Lanes 3 and 4), and run on a 1.8% formaldehyde-agarose gel to visualize the RNA species. The large RNA species are being removed with no contaminating large RNA present in the microRNA elution. Lane M is Norgen's 1 kb RNA Ladder.

In order to assess the biological activity of the RNAs isolated, RT-PCR was performed. Unlike regular RT-PCR, the amplification and detection of small RNA molecules such as miRNA requires the addition of an adaptor. One of the commonly-used protocols involves the addition of a poly(A) tail to the miRNA by Poly(A) Polymerase (5). This method was used, and Figure 5 (Panel A) shows the amplification of the *miR-21* transcript from small RNAs isolated by Norgen's microRNA Purification Kit. The PCR product of 69 bp (miRNA transcript size = 23 nucleotides and adaptor size = 46 nucleotides) was detected in both the small RNA fractions (Lanes 2 and 3) as well as the total RNA control isolated using Norgen's Total RNA Purification Kit (Lane 1).

Similarly, the 5S rRNA (Figure 5, Panel B), which is commonly used as a loading control for miRNA RT-PCR, was amplified from the purified small RNA. This suggested that the small RNA isolated was of a high purity and had retained its biological activity.

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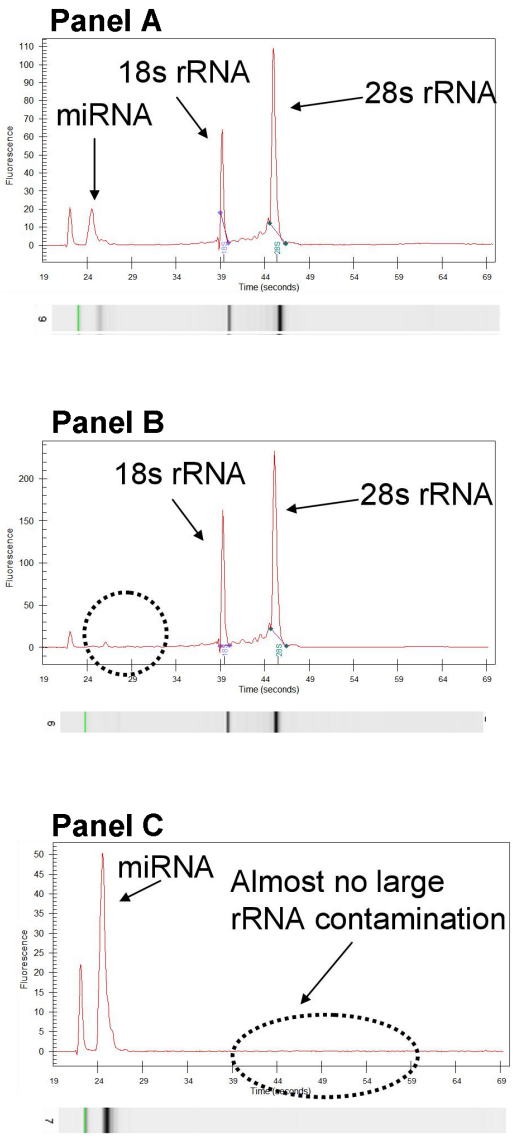

Figure 4. Efficient Removal of Large RNA Species. Different RNA species were isolated from HeLa cells, resolved on an Agilent® Lab-On-A-Chip and electropherograms were generated. Panel A contains all the RNA species present in HeLa cells as isolated with Norgen's Total RNA Kit, and acts as a control. Panel B and C contain RNA that was isolated using Norgen's microRNA Purification Kit. Panel B shows the large RNA species removed using the Large RNA Removal Columns, and Panel C shows the microRNA that is isolated using the microRNA Enrichment Columns. Thus there is no contamination of the microRNA with any large RNA species.

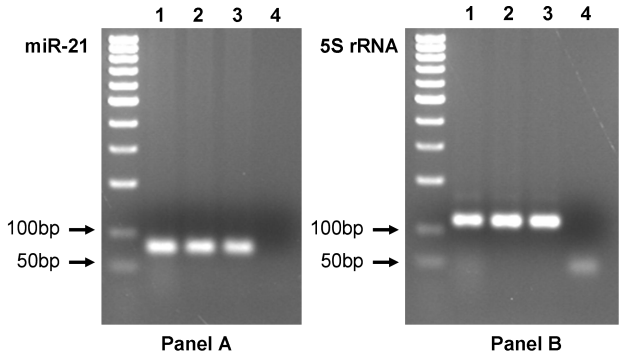

Figure 5. RT-PCR Results for microRNA Isolated Using Norgen's Kit. Panel A shows the amplification of the *miR-21* transcript from small RNAs while Panel B shows the 5S rRNA amplification from small RNAs. Lane 1 in both panels shows the results when total RNA isolated from HeLa cells using Norgen's Total RNA Purification Kit was used as a control. Lanes 2 and 3 contain the successful RT-PCR when the microRNA isolated using Norgen's microRNA Purification Kit was used as the template, and Lanes 4 contain the non-template control. The RT-PCR was successful for both reactions using the microRNA as the template.

CONCLUSIONS

Through the analysis of the performance of Norgen's microRNA Purification Kit for isolating small RNA from HeLa cells, a number of conclusions regarding Norgen's kit can be made:

1. Norgen's kit allows for the isolation of high quality small RNA within 20 minutes and without the use of any organic solvent. Norgen's kit protocol requires no organics and has a minimal number of steps, thus providing a more convenient method than some existing commercial kits.

2. Norgen's kit isolates small RNAs of high purity and integrity. The high quality and integrity of the RNA is demonstrated in Figures 2 to 4. Figure 2 shows that the RNA isolated is truly <200 nucleotides and shows no degradation. Figures 3 and 4 show that Norgen's kit isolates small RNAs that are free of any large RNA contamination.

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3. Norgen's kit isolates small RNAs, including miRNA that are fully compatible with downstream applications. The compatibility with downstream applications is demonstrated in Figures 4 and 5. Figure 4 shows that the small RNAs can be resolved on a Lab-on-A-Chip, allowing proper quantification of the small RNA. Figure 5 shows that the small RNAs can be modified by enzymes such as Poly(A) Polymerase, and subsequently used for RT-PCR amplification of miRNAs.

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