High-Throughput Purification of Total RNA Including miRNA without the use of Phenol

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INTRODUCTION

The isolation of high quality total RNA is an important first step in a number of downstream applications including RT-PCR, Northern blotting, cDNA library construction, *in situ* hybridization, primer extension and microarray gene expression analyses. The need for high quality RNA rapidly from large numbers of samples generates a crucial requirement for high-throughput methods of RNA purification. Currently, most commercial RNA purification products utilize silica membranes as an affinity matrix, which require an additional phenol:chloroform extraction step in order to purify true total RNA, including small RNAs. This organic extraction step increases the difficulty of the RNA purification, and results in an inconvenient method of high throughput isolation of true total RNA using the 96-well format.

Norgen's Total RNA Purification 96-Well Kit provides a rapid method for the high-throughput isolation and purification of total RNA from cultured animal cells, tissue samples, blood, bacteria, yeast, fungi and plants. The kit purifies all sizes of RNA, from large mRNA and ribosomal RNA down to microRNA and small interfering RNA without the use of phenol of chloroform, therefore resulting in a convenient high-throughput method for total RNA purification. The purified RNA is of the highest integrity, and can be used in a number of downstream applications.

Purification is based on 96-well column chromatography using Norgen's proprietary resin as the separation matrix. The RNA is preferentially purified from other cellular components such as proteins without the use of phenol or chloroform. The purification could be performed on either a vacuum manifold or using centrifugation (**Figure 1**). The process involves first lysing the cells or tissue of interest with the provided Lysis Solution. Ethanol is then added to the lysate, and the solution is loaded onto the 96-Well Filter Plate. Norgen's resin binds RNA in a manner that depends on ionic concentrations, thus only the RNA will bind to the resin in the wells, while the contaminating proteins will be removed in the flow-through or retained on the top of the resin. The bound RNA is then washed with the provided Wash Solution in order to remove any remaining impurities, and the purified total RNA is eluted with the Elution Buffer. The purified RNA is of the highest integrity, and can be used in a number of downstream applications.

This application note describes a high-throughput method of isolating RNA using Norgen's Total RNA Purification 96-Well Kit. Various parameters, including reproducibility and consistency of RNA quantity and quality, as well as the speed of the purification process are analyzed in depth.

MATERIALS AND METHODS

RNA Isolation

Total RNA was isolated from different cell types using Norgen's Total RNA Purification 96-Well kit according to the provided protocol (**Figure 1**). RNA was isolated from each of the following: 5 x 10^8 CFUs *E. coli*; 7.5 x 10^5 HeLa or CHO cells and; 8 mg of brain, kidney, liver, lung and spleen tissues. In brief, lysate was prepared according to the cell or tissue type. The remaining steps of the procedure for the purification of total RNA was the same for all the different types of lysate. A 96-well filter plate was placed on top of an RNAse-free 96-well plate, 500 µL of lysate + ethanol was applied into each well and the assembly centrifuged at 3,000 *x g* for 2 minutes. The flowthrough was discarded and 400 µL of Wash Solution applied to each well. The assembly was again centrifuged at 3,000 *x g* for 2 minutes. The flowthrough was again discarded and the washing step repeated an additional two times. The 96-well filter plate was then dried by centrifuging at 3,000 *x g* for 5 minutes. For elution, the 96-well filter plate was placed on top of an elution plate and 75 µL of elution buffer was added to each well and the assembly centrifuged at 3,000 *x g* for 2 minutes.

In addition to the above isolations, RNA was also purified from HeLa cells (7.5 x 10^5) using the leading market competitor's RNA 96-well kit according to the manufacturer's methods. Purified RNA was then used in comparative analyses.

RNA Gel Electrophoresis

The purified RNAs were run on 1X MOPS, 1.5% formaldehyde-agarose gels for visual inspection. In general, 5 µL of each 100 µL elution was run on the gel.

RT-qPCR Assays

RNA purified from HeLa cells was used as the template in an RT-qPCR reaction, using primers specific for the *S15* gene. microRNAs purified from HeLa cells were modified according to (1) 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 amplificaiton, primers specific for the human *miR-21* (5' CGTGACGTTAGCTTATCAGACTG 3') and the adaptor (according to (1)) were used. The sensitivity of the RNA isolation protocol was further demonstrated by using varying amounts of HeLa cells for sample preparation prior to RT-qPCR. The input number of HeLa cells were as follows: 1 x 10^5, 1 x 10^4, 1 x 10^3, 1 x 10^2, 1 x 10^1 and 1 x 10^0. Total RNA purified from each input level was then used as template in an RT-qPCR reaction again using primers specific for the *S15* gene.

RESULTS AND DISCUSSION

RNA was isolated from 5 x 10^8 *E. coli* cells using Norgen's Total RNA Purification 96-Well Kit according to the provided protocol as described in **Figure 1**. The entire protocol was completed in 30 minutes. RNA samples were then run on 1X MOPS, 1.5% formaldehyde-agarose gels for visual inspection (**Figure 2**). The isolated RNA samples were of a high quality with good yield and size diversity, including small RNAs.

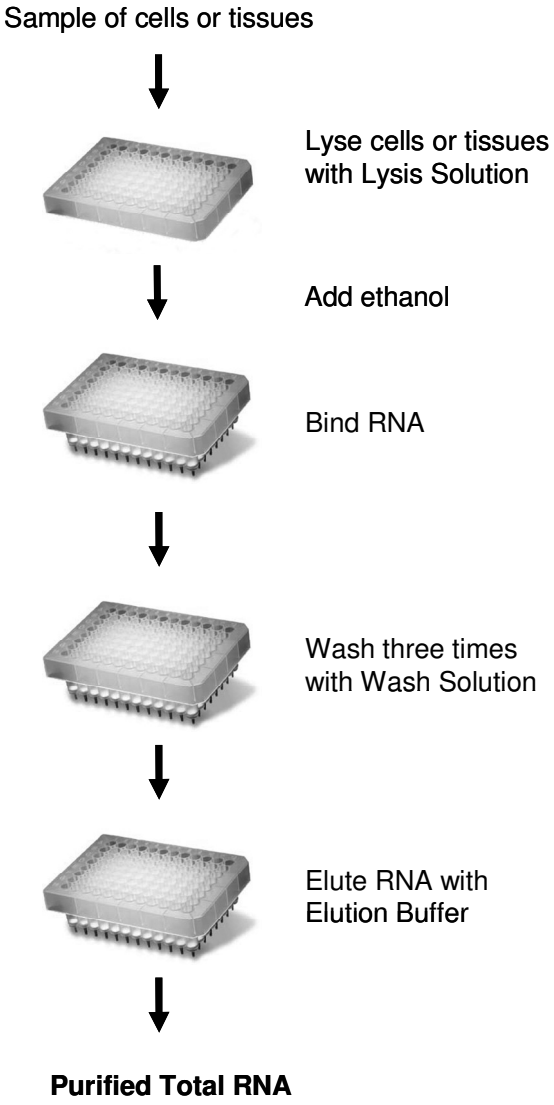

Figure 1. Procedure Flowchart for the Purification of Total RNA using Norgen's Total RNA Purification 96-Well Kit.

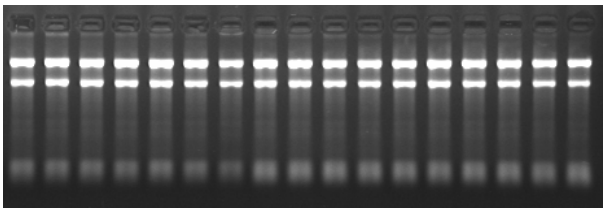

Figure 2. Consistent Isolation of True Total RNA. Total RNA was isolated from 5 x 10^8 *E. coli* cells using Norgen's Total RNA Purification 96-Well Kit. Seventeen replicates of the isolation were performed. Norgen's kit consistently isolated high quality true total RNA with a high yield and size diversity, including small RNA species.

A commercially available competitor's 96-well RNA purification kit was also used for comparison. Total RNA was isolated from 7.5 x 10^5 HeLa cells using Norgen's kit as well as the competitor's kit (**Figure 3**). Purified RNAs were run on a 1X MOPS, 1.5% formaldehyde-agarose gel for visual inspection and comparison. RNA samples prepared using Norgen's kit were of a high quality and had a higher yield when compared to the RNA samples purified using the competitor's kit. In addition, true total RNA, including microRNA, was observed on the gel for the RNA samples purified by Norgen's kit. In contrast, the competitor's kit failed to isolate small RNAs. Norgen's purification kit truly isolates total RNA with a wider size diversity and quality then the leading market competitor.

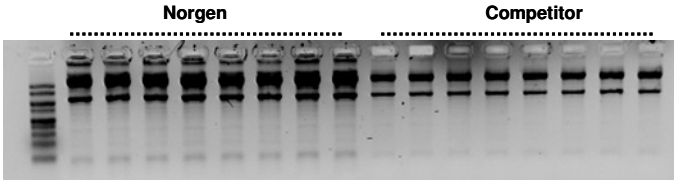

Figure 3. Resolution of HeLa RNA on a 1X MOPS, 1.5% Formaldehyde-Agarose Gel and Comparison to Market Competitor. Total RNA was isolated from 7.5 x 10^5 HeLa cells using Norgen's Total RNA Purification 96-Well Kit and a leading market competitor's 96-well RNA purification kit. Eight replicates were performed for each kit. While both kits consistently isolated RNA, Norgen's kit isolated total RNA with an improved yield and size diversity when compared to the competitor.

In addition to bacterial and HeLa cells, total RNA was also purified from 8 mg of brain, kidney, liver, lung and spleen tissues, as well as CHO cells using Norgen's Total RNA Purification 96-Well Kit (**Figure 4**). The RNA samples were again visualized by running on a 1X MOPS, 1.5% formaldehyde-agarose gel. Norgen's kit allowed the isolation of RNA from all sample types. Norgen's kit can therefore be used to successfully isolate total RNA, including small RNA species, from a wide variety of sample types.

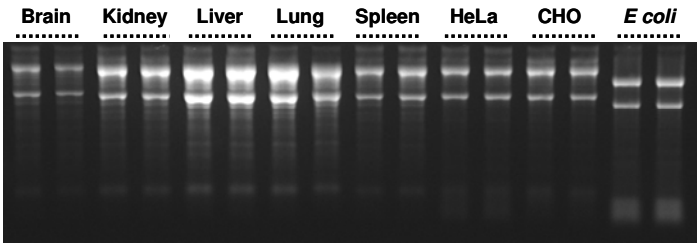

Figure 4. Isolation of Total RNA from a Diversity of Cell and Tissue Types. Total RNA was isolated from 8 mg of brain, kidney, liver, lung and spleen tissues, 7.5 x 10^5 HeLa and CHO cells and 5 x 10^8 bacteria using Norgen's Total RNA Purification 96-Well Kit. As can be observed on the formaldehyde-agarose gel, Norgen's kit can be used to successfully isolate total RNA, including small RNA species, from a broad range of sample types.

In order to analyze the biological activity of the total RNA purified using Norgen's Total RNA Purification 9-Well Kit, RT-qPCR reactions were performed. The first RT-qPCR reaction was for the amplification of the mRNA *S15* transcript, and the other RT-qPCR reaction was for the microRNA transcript *miR-21*. The RT-qPCR reactions were performed on 11 different replicates, and graphs plotting the Ct values for each of the replicates were also generated. **Figure 5** (**Panel A**) shows the amplification of the *S15* transcript from total RNA isolated from 7.5 x 10^5 HeLa cells using Norgen's kit. The PCR product was consistently isolated in all replicate samples, as indicated by graphing the Ct values for all the replicates (**Figure 5, Panel C**). This suggests that Norgen's Total RNA Purification 96-Well Kit can consistently isolate RNA of a high purity and which has retained its biological activity.

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 (1). This method was employed here, and **Figure 5 (Panel B)** demonstrates the amplification of the *miR-21* transcript from microRNA purified using Norgen's kit. The PCR product was successfully detected from the total RNA purified from 7.5 x 10^5 HeLa cells. In addition, the *miR-21* microRNA was amplified in a consistent manner from all sample replicates, with low variability of the Ct values, similar to what was observed for the mRNA (Figure 5, Panel D). Therefore Norgen's Total RNA Purification 96-Well Kit consistently isolates both types of RNA.

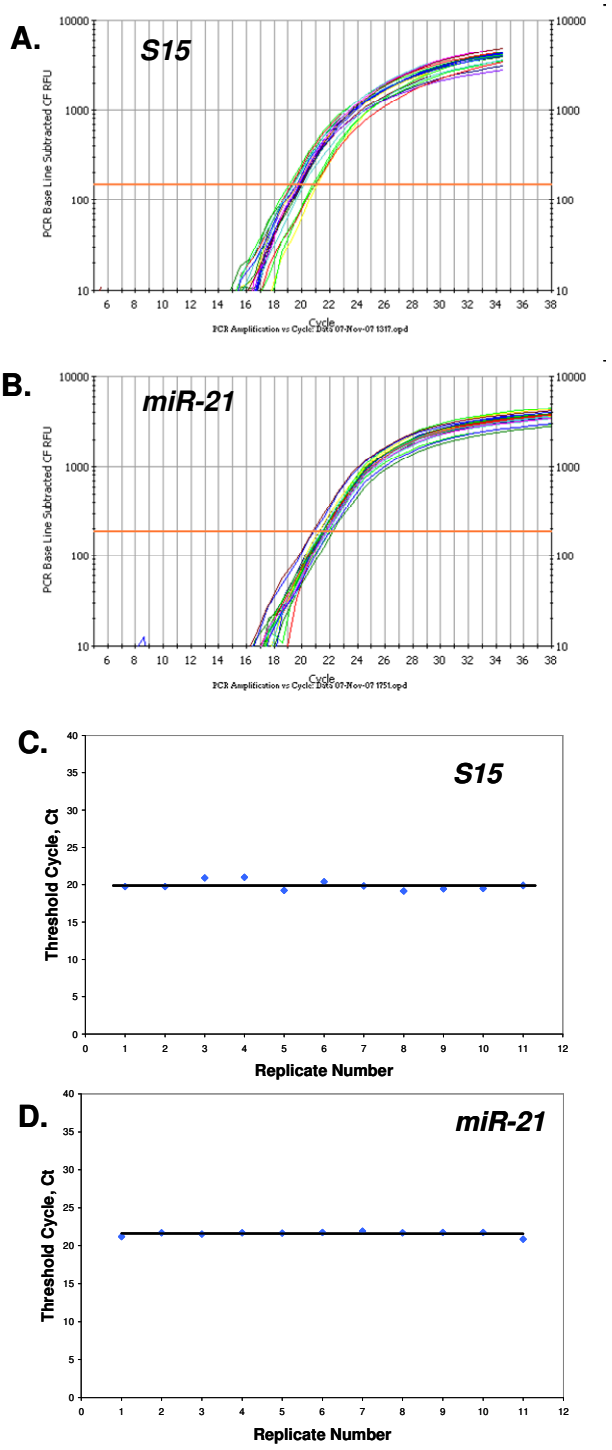

Figure 5. Consistent Isolation of Total RNA. RNA was isolated from samples of 5 x 10^5 HeLa cells using Norgen's kit, and aliquots of each total RNA sample were used as the template in two different RT-qPCR reactions. In the first RT-qPCR the *S15* gene of mRNA was amplified (Panel A), and in the second RT-qPCR reaction the *miR-21* microRNA was amplified (Panel B). Both the mRNA and microRNA were amplified in a consistent manner from all the samples, with low variability of the Ct values (Panels C and D). Thus both types of RNA are being consistently isolated using this kit.

The sensitivity of detection was then determined for total RNA purified using Norgen's Total RNA Purification 96-Well Kit. A total of six input amounts of HeLa cells were tested: 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0. Purified RNA was then used as the template in RT-qPCR with primers specific for the *S15* gene. As shown in **Figure 6,** total RNA that maintained its biological activity was detected for all input levels. The total RNA was isolated and detected linearly from as little as a single cell. Norgen's Total RNA Purification 96-Well Kit can therefore be used for the highly sensitive detection of RNA.

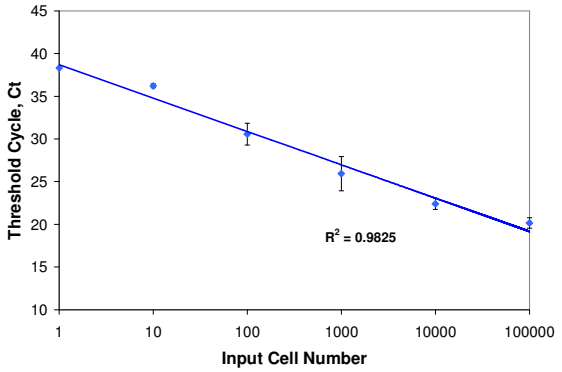

Figure 6. Sensitive RNA Isolation to <10 Cells. RT-qPCR was used to detect mRNA isolated from various input amounts of HeLa cells using Norgen's Total RNA Purification 96-Well Kit. The primers used for the RT-qPCR detected the *S15* gene. Total RNA was isolated and detected linearly from as little as a single HeLa cell.

CONCLUSIONS

Through the analyses of the performance of Norgen's Total RNA Purification 96-Well Kit, a number of conclusions regarding the benefits of Norgen's kit can be made:

1. Norgen's high-throughput kit allows for the isolation of up to 96 high quality total RNA samples within 30 minutes and without the use of any organic solvent. Total RNA, including all small RNA species, are isolated without the use of harmful chemicals such as phenol or chloroform. As Norgen's kit protocol eliminates the use of organics and has a minimal number of steps, it is a more convenient method than other commercial kits.

2. The 96-well kit allows the isolation of a diversity of RNA species. All RNA species can be isolated, from large mRNA and ribosomal RNA down to microRNA and small interfering RNA. These small RNAs maintain their biological activity and can be modified by enzymes such as Poly(A) Polymerase and subsequently used for RT-PCR amplification.

3. Isolation of RNA from a wide variety of samples can be carried out using Norgen's kit. RNA can be isolated from cultured animal cells, small tissue samples and bacteria (Figure 4).

4. Isolation of RNA can be successfully carried out from very small samples. Total RNA has been isolated and detected from as little as a single animal cell (Figure 6) indicating the sensitivity of the sample processing method and suitability of the purified RNA for downstream assays.

5. Fast and easy processing. Each 96-well plate can be rapidly processed in 30 minutes.

6. Recovered RNA is suitable for many downstream applications. Purified RNA is of a high quality and maintains its biological activity. As a result it can be used in a number of downstream applications including real-time PCR, reverse transcription PCR, Northern blotting, RNAse protection and primer extension and expression array analyses requiring the use of intact RNA.

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