

Rapid Method for the Purification of Total RNA from Formalin-Fixed Paraffin-Embedded (FFPE) Tissue Samples

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INTRODUCTION

It is estimated that there are currently more than a billion tissue samples archived in hospitals and tissue banks around the world, and the vast majority of these are formalin-fixed and paraffin-embedded (FFPE) tissue samples (1). These tissues represent a largely untapped resource for molecular profiling of clinical samples and biomarker discovery. The major problem with these samples is that while the process of formalin-fixing and paraffin-embedding ensures that the tissues are well preserved, the difficulty lies in extracting high quality biomolecules from these samples that can be used in downstream applications.

Norgen's FFPE RNA Purification Kit provides a rapid method for the isolation and purification of total RNA (including microRNA) from formalin-fixed paraffin-embedded (FFPE) tissue samples. Using formalin to fix tissues leads to cross-linking of the RNA and proteins, and the process of embedding the tissue samples can also lead to fragmentation of the RNA over time. Norgen's FFPE RNA Purification Kit provides conditions that allow for the partial reversing of the formalin modifications, resulting in a high quality and yield of RNA. The kit is able to purify all sizes of RNA, from large mRNA and ribosomal RNA down to microRNA (miRNA) and small interfering RNA (siRNA), depending on the age of the FFPE tissue as fragmentation of the RNA is known to occur over time.

With Norgen's FFPE RNA Purification Kit, the purification is based on spin column chromatography using Norgen's proprietary resin as the separation matrix. The RNA is preferentially purified from other cellular components without the use of phenol or chloroform. The purified RNA is of the highest integrity, and can be used in a number of downstream applications including RT-qPCR, reverse transcription PCR, primer extension, expression array assays and microarray analyses.

This application note describes a rapid method of isolating and purifying total RNA from FFPE kidney tissue samples using Norgen's FFPE RNA Purification Kit. FFPE RNA was also purified using leading competitor's kits for comparison. The quality of the purified RNA samples and their suitability in downstream assays is then analyzed in depth.

MATERIALS AND METHODS

RNA Isolation

The procedure for purifying total RNA using Norgen's FFPE RNA Purification Kit is shown in **Figure 1**. Briefly, an FFPE kidney tissue block was first de-paraffinized by adding 1 mL of xylene to the sample and incubating at 50°C for 5 minutes. The sample is then centrifuged at 14,000 x g for 2 minutes and the xylene carefully removed. Next, 1 mL of ethanol is added to the pellet and mixed by vortexing. The sample is again centrifuged at 14,000 x g for 2 minutes and the ethanol carefully removed. The ethanol step is repeated and then the pellet allowed to thoroughly air dry. Lysate is then prepared by the addition of 300 µL of Digestion Buffer and 10 µL of Proteinase K solution. The mixture is then incubated at 55°C for 15 minutes, followed by an incubation at 80°C for 15 minutes. Following digestion, 300 µL of Binding Solution is added and mixed along with the addition of 600 µL of 95% ethanol. Next, 600 µL of the lysate is then applied to an assembled spin column and centrifuged for one minute. The flowthrough is discarded and the remaining lysate is similarly loaded onto the column. The column is then washed three times with 400 µL of Wash Solution. The column is dried by centrifuging for 2 minutes. For elution, the column is placed into a new 1.7 mL elution tube and 50 µL of RNA Elution Buffer is added to the column. The column assembly is then centrifuged at 200 x g for 2 minutes followed by a 1 minute spin at 14,000 x g.

In addition, RNA was also purified from FFPE tissue blocks using leading market competitors kit according to the manufacturer's methods. The 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 50 µL elution was run on the gel.

Capillary Electrophoresis

The purified RNAs were loaded onto an Agilent[®] RNA Nano 6000 chip and resolved on an Agilent[®] 2100 BioAnalyzer according to the manufacturer's instructions.

RT-qPCR Assays

RNA purified from FFPE kidney tissue blocks was used as the template in RT-qPCR reactions using primers specific for either the *β-actin* gene, 5S rRNA or *mi-R21* microRNA. The microRNAs were modified according to (2) 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 (3). 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 (3)) were used.

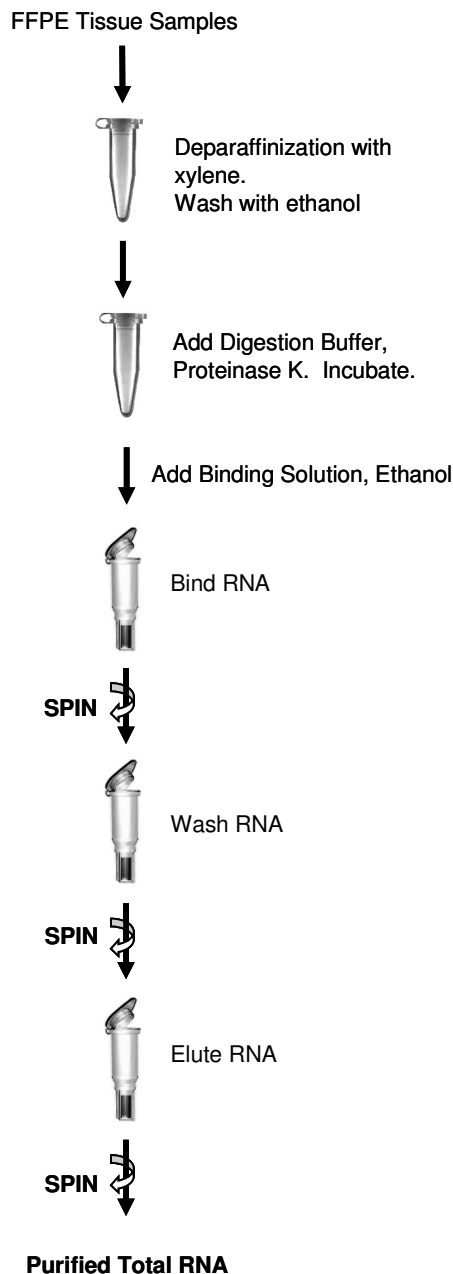


Figure 1. Procedure flowchart for the purification of total RNA using Norgen's FFPE RNA Purification Kit.

RESULTS AND DISCUSSION

RNA was isolated from 20 μm sections of kidney FFPE tissue blocks using Norgen's FFPE RNA Purification Kit according to the provided protocol as described in **Figure 1**. In addition, RNA was isolated from 20 μm sections of kidney FFPE tissue blocks using the leading market competitor's kit. The RNA samples were then run on 1X MOPS, 1.5% formaldehyde-agarose gels for visual inspection (**Figure 2**). From observing the gel, it can be seen that the RNA isolated using Norgen's kit was of a higher quality and yield than the RNA isolated using the competitor's kit. Furthermore, the total RNA isolated using Norgen's kit had a greater size diversity, including small RNAs.

The quality of RNA isolated by Norgen's FFPE RNA Purification Kit and the competitor's kit was further analyzed by capillary gel electrophoresis (**Figure 3**). **Figure 3** demonstrates that when the FFPE total RNA isolated using Norgen's kit was resolved on an Agilent Lab-on-a-Chip all the RNA species, including microRNA, 18S rRNA and 28S rRNA can be observed. More importantly, Norgen's kit produced an RNA sample with both a higher yield and purity than did the leading market competitor's kit, as indicated by the higher RIN value. Furthermore, the results in **Figure 3** show the compatibility of the small RNAs isolated with Norgen's kit with the Bioanalyzer. This provides added benefits for quantifying the isolated microRNA, as spectrophotometry may not be applicable for quantifying small RNA, particularly when low input amounts are used.

In order to assess the biological activity of the RNAs isolated, RT-qPCR was performed. Total RNA was isolated from equal amounts of an FFPE kidney tissue sample using Norgen's FFPE RNA Purification Kit and a leading competitor's kit. The purified RNA was then used as the template in RT-qPCR reactions for detection of the *β -actin* gene (**Figure 4**). From observing **Figure 4** it can be seen that the RT-PCR was successful, indicating the high quality of the RNA isolated using Norgen's kit. Furthermore, Norgen's kit isolated higher yields of RNA, as indicated by the lower C_T values.

The total kidney RNA purified using Norgen's kit and the competitor's kit was also used as a template in an RT-qPCR reaction to detect *miR-21* microRNA (**Figure 5**). 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 (2). This method was used, and **Figure 5** shows the amplification of the *miR-21* transcript from small RNAs isolated by Norgen's kit and the competitor's kit. Again, Norgen's kit isolated higher yields of RNA as indicated by the lower C_T values in the RT-PCR reactions. In addition, the successful RT-qPCR reaction shows that Norgen's kit isolated not only the large RNA but also microRNA, indicating the diversity of RNA species isolated using Norgen's FFPE RNA Purification Kit. Furthermore, these small RNAs maintain their biological activity and can be modified by enzymes such as Poly(A) Polymerase and subsequently used for RT-qPCR reactions.

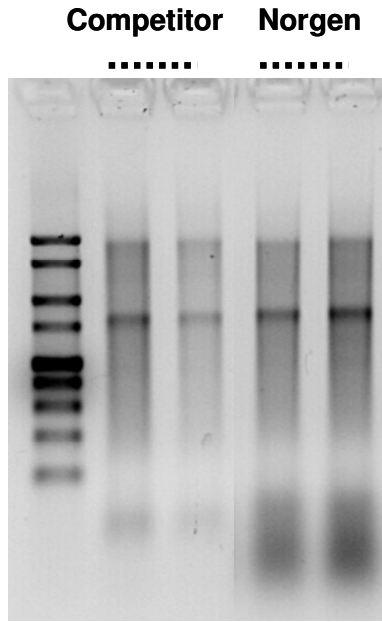


Figure 2. Resolution of FFPE RNA on a 1X MOPS, 1.5% formaldehyde-agarose gel.

Total RNA was purified from 20 μm sections of FFPE kidney tissue samples using Norgen's FFPE RNA Purification Kit and a competitor's kit, and were loaded on a 1X MOPS 1.5% formaldehyde gel (5 μL of the 50 μL elutions) for visualization. Isolations were performed in duplicate for each kit. Norgen's kit consistently isolated RNA with a higher yield and greater size diversity. Lane 1 contains Norgen's 1Kb RNA Ladder.

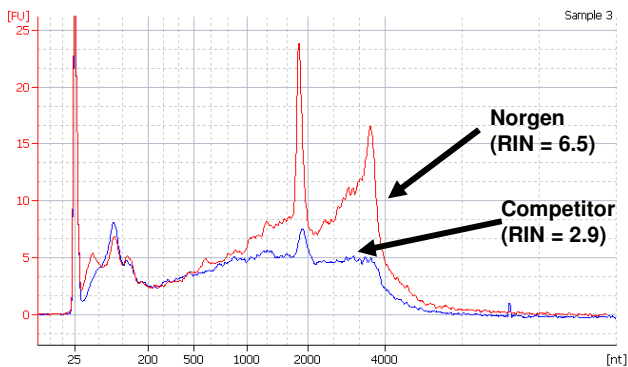


Figure 3. High Quality and Yield of RNA Resolved on the Agilent BioAnalyzer.

Total RNA was isolated from equal amounts of an FFPE tissue sample using Norgen's FFPE RNA Purification Kit and a leading competitor's kit. The purified RNA was then resolved on an Agilent BioAnalyzer. As can be seen, Norgen not only isolated higher yields of total RNA, but the RNA was also of a higher quality as evidenced by the higher RIN values obtained with Norgen's RNA.

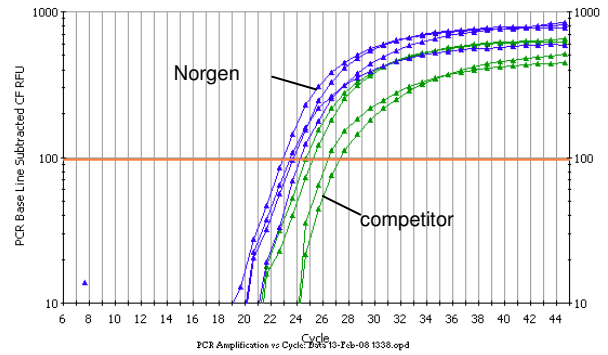


Figure 4. RT-qPCR of FFPE Kidney RNA Using Primers Specific for the β -actin Gene.

Total RNA was isolated from equal amounts of an FFPE tissue sample using Norgen's kit and a leading competitor's kit. The purified RNA was then used as the template in an RT-qPCR reaction for detecting the β -actin gene. The amplification was successful, indicating the high quality of Norgen's purified FFPE RNA. Furthermore, Norgen's kit isolated higher yields of RNA as indicated by the lower C_T values.

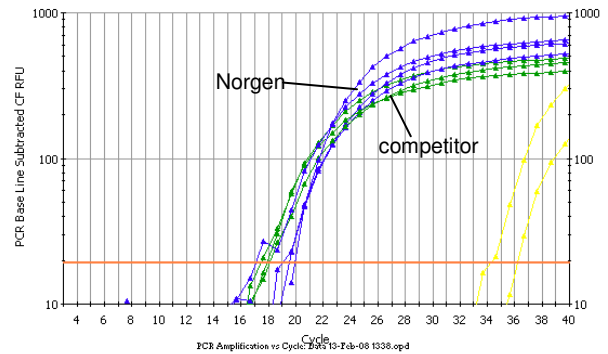


Figure 5. Detection of microRNA in FFPE Kidney Total RNA using RT-qPCR.

Total RNA was isolated from equal amounts of an FFPE tissue sample using Norgen's kit and a leading market competitor. The purified RNA was then used as the template in an RT-qPCR reaction for the detection of microRNA (*miR-21*). Norgen's kit was able to successfully isolate total RNA, including microRNA. Furthermore, Norgen's kit isolated higher yields of RNA as indicated by the lower C_T values.

Next, total RNA was isolated from equal amounts of an FFPE kidney tissue sample using Norgen's FFPE RNA Purification Kit and two different competitor's kits according to the manufacturer's instructions. The RNA yield obtained from the 3 kits was then compared using spectrophotometric analyses. Similar to what was observed on agarose gels, RNA purified using Norgen's kit had a much higher yield than the two market competitors (**Figure 6**).

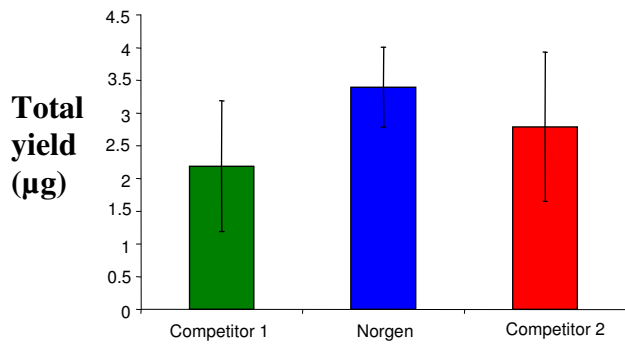


Figure 6. Comparison of Total RNA Yield

Total RNA was isolated from equal amounts of FFPE kidney tissue using Norgen's FFPE RNA Purification Kit and two leading competitor's kits. The graph demonstrates the mean yield of RNA for 18 sample replicates for each kit, and the vertical bars represent the standard deviation. Norgen's kit consistently purified total RNA with a higher yield than was obtained using the market competitor's kits.

In order to assess the quality of the RNA isolated from FFPE samples using Norgen's FFPE RNA Purification Kit and two leading competitor's kits, RT-qPCR was again performed (Figure 7). A primer set for detection of the *β-actin* gene was used for the amplification and detection of mRNA (Figure 7, Panel C). In addition, primers specific for *miR-21* were used for the detection of microRNA in the purified RNA samples (Figure 7, Panel A). Similarly, the *5S rRNA* (Figure 7, Panel B), which is commonly used as a loading control for miRNA RT-PCR, was amplified from the purified small RNA. The results of 18 different amplifications using each set of primers were then graphed in terms of the mean ΔCt value. Norgen's kit consistently isolated RNA of a higher yield than that obtained by the two leading market competitors, as indicated by the higher ΔCt value in each case. Furthermore, not only were the large RNA species detected, but the small RNA species were also detected from the Norgen purified samples, indicating the diversity of the RNA species isolated using Norgen's kit. This further suggested that all species of RNA isolated using Norgen's kit were of a high purity and had retained their biological activity.

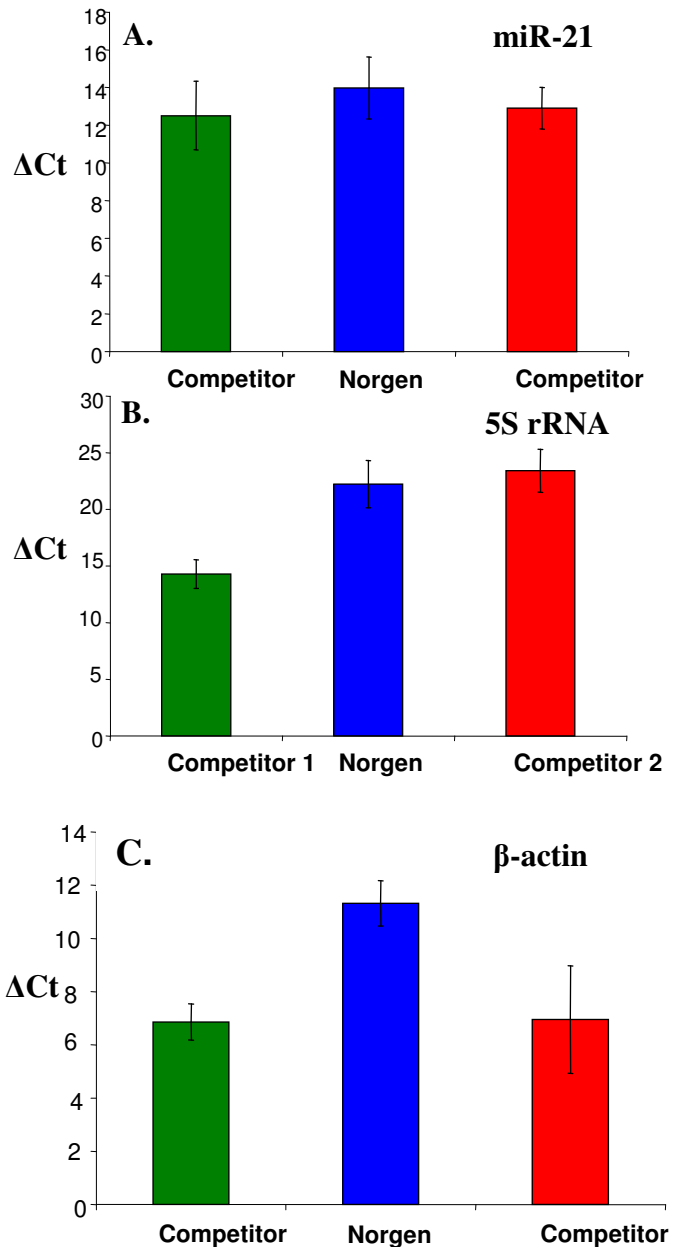


Figure 7. RT-qPCR Results with Total RNA Purified by Norgen's Kit and Two Market Competitors. The graphs demonstrate the mean ΔCt value for 18 sample replicates isolated using Norgen's FFPE RNA Purification Kit (blue bar), and two leading competitors kits (green bar and red bar). Panel A is the results from RT-qPCR using *miR-12* primers, Panel B is the results from RT-qPCR using *5S rRNA* primers, and Panel C is the results from using the *β-actin* primers. The vertical bars represent the standard deviation. Norgen's kit consistently isolated RNA of a higher yield than that obtained by the two leading market competitors. Also, both the large RNA species (rRNA and mRNA) and the small RNA species (microRNA) were detected in Norgen's total RNA samples, indicating the diversity of RNA species isolated with Norgen's kit.

CONCLUSIONS

Through the analyses of the performance of Norgen's FFPE RNA Purification Kit, a number of conclusions regarding the benefits of the kit can be drawn:

1. Norgen's FFPE RNA Purification Kit allows for the isolation of total RNA using a rapid procedure.

Isolation of total RNA from FFPE tissue sections using a rapid spin column format.

2. Isolation of a diversity of RNA species. All RNA species can be isolated, from large mRNA and ribosomal RNA down to microRNA. These small RNAs maintain their biological activity and can be modified by enzymes such as Poly(A) Polymerase and subsequently used for RT-qPCR reactions.

3. Isolation of RNA can be successfully carried out from very small samples. Total RNA was isolated and detected from as little as a 20 μ m section of kidney FFPE tissue blocks, indicating the sensitivity of the sample processing method.

4. High quality and integrity of the isolated RNA. Purified RNA is of 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.

5. High yields. Norgen's FFPE RNA Purification Kit allows for the purification of high yields of total RNA. Norgen's kit consistently isolated higher yields of RNA than the leading competitor's kits.

6. No organic solvents. Total RNA is isolated from FFPE tissue samples without the use of harmful chemicals, such as phenol or chloroform.

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