Evaluation of Different Extraction Methods for Recovering Nucleic Acids from Formalin-Fixed Paraffin-Embedded Tissue Samples

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Abstract

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. 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. The ability to purify RNA and DNA from such samples could contribute tremendously to the study of transcriptomes and genomes of individuals carrying different medical conditions including genetic and infectious diseases. This study compared the performance of a number of commercially available products designed specifically for DNA or RNA isolation from FFPE samples. For extraction of total RNA (including microRNA) from FFPE samples, several column-based methods were tested. All methods showed similar yield and quality of the RNA extracted. However, based on RT-qPCR study, all silica-based column protocols lost significant sensitivity to larger RNA at the condition required to recover small RNAs. In contrast, a silicon carbide-loaded column protocol showed no bias of RNA size and had good affinity for all sizes of RNA, from large mRNA to microRNAs. For extraction of DNA from FFPE samples, kit performance (based on DNA quantity, quality and performance in qPCR) were very similar, although the heat reversal of formalin cross-link appeared to be the most critical factor for successful isolation. In summary, the type of affinity medium played an important role in unbiased RNA recovery from FFPE samples, which may greatly affect downstream transcription assays and hence biomarker discover

Introduction

- Formalin-fixed, paraffin-embedded tissues (FFPE) represent a largely untapped resource for biomarker discovery, single nucleotide polymorphism (SNP) arrays[1], and forensic research[2].
- There are currently more than a billion FFPE tissue samples that have been archived in hospitals and tissue banks[3], and FFPE tissues are the most widely used sample type for retrospective studies[4].
- Despite the vast potential for FFPE tissues, the quality of DNA and RNA isolated from these samples has been argued to be too low for reliable gene expression profiling[5].
- 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.
- The FFPE nucleic acid extraction procedure can often be the deciding factor in the success of many subsequent downstream applications, including real-time polymerase chain reaction (qPCR). While the formalin fixation itself has been found to cause nucleic acid denaturation[2], it has also been found to cause PCR inhibition or failure due to formalin-DNA or formalin-RNA interactions[2].
- FFPE tissues are sometimes the only resource available, and thus a reliable nucleic acid extraction method is of utmost importance[2].

Objectives of the Study

1) To compare three column-based systems for their ability to isolate RNA from FFPE tissues, with emphasis placed on yield, quality and RT-qPCR performance.
2) To compare silica matrix columns to a silicon carbide matrix for their ability to recover RNA and DNA without fragment size bias.
3) To demonstrate the effect of heating during deparaffinization on DNA recovery from FFPE tissues.

Materials and Methods

RNA Isolation. Total RNA was isolated from 20 µm sections of an FFPE kidney tissue block using three kits, as per the manufacturer's protocols:
1) Norgen's FFPE RNA Purification Kit
2) Ambion's® RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE
3) Qiagen's miRNeasy FFPE Kit

RNA Gel and Capillary Electrophoresis. The purified RNAs were run on 1X MOPS, 1.5% formaldehyde-agarose gels for visual inspection, using 5 µL of each 50 µL elution. The purified RNAs were also loaded onto an Agilent® RNA Nano 6000 chip and resolved on an Agilent® 2100 BioAnalyzer according to the manufacturer's instructions.

RT-qPCR Analysis. The purified RNA was then used as the template in RT-qPCR reactions using primers specific for either the β-actin gene, 5S rRNA or miR-21 microRNA. The microRNAs were modified according to Shi and Chiang (2005)[6] 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 for all 3 genes using Invitrogen's Superscript II system and a poly(T) adaptor primer[7]. The cDNAs were then used as the template in qPCR reactions. Additionally, the same samples were run in reactions containing no reverse transcriptase to generate ΔCt values, used to determine amount of genomic DNA contamination in samples.

DNA Isolation. DNA was isolated from 20 µm sections of an FFPE kidney tissue block using two kits according to the manufacturer's protocol:
1) Norgen's FFPE RNA/DNA Purification Kit
2) Qiagen's QIAamp DNA FFPE Tissue Kit

DNA Gel Electrophoresis and Spectrophotometry. The purified DNA from the heating experiment was run on a 1X TAE, 3% agarose DNA gel for visual inspection, using 15 µL of each 150 µL elution run at 150V for 30 min. DNA quantification was performed using Nanovue Plus (GE Healthcare) spectrophotometry.

qPCR Analysis. The purified DNA from both the heating experiment and the comparison between competitor kits was used as the template in a qPCR reaction using primers specific for the β-actin gene.

Results and Discussion

1- Comparison of Quantity and Quality of RNA Using the Norgen and Ambion Systems. Norgen's FFPE RNA kit was compared to Ambion's kit. Norgen's kit was found to consistently isolate RNA with a higher yield and greater size diversity than Ambion's kit. This is due to Norgen's patented technology (silicon carbide columns), which differs from competitor technology (silica columns), allowing for total RNA isolation from FFPE tissues, without size bias. Norgen was found to consistently isolate RNA of the highest yield (Figure 1A) and quality, as evidenced by both higher RIN values (Figure 1B) and lower Ct values (Figures 1C and 1D). Ct values generated from RT-qPCRs are dependent on the quality of the RNA sample, as well as the concentration of RNA. A high quality RNA sample will have lower Ct values than a sample of lower quality. When equal amounts of RNA were used, Norgen's kit consistently resulted in lower Ct values compared to Ambion's kit. While Norgen and Ambion isolate similar miRNA yields (Figure 1C), dramatic differences can be seen in large RNA recovery (Figure 1D), where Norgen was found to recover higher amounts of large RNA species.

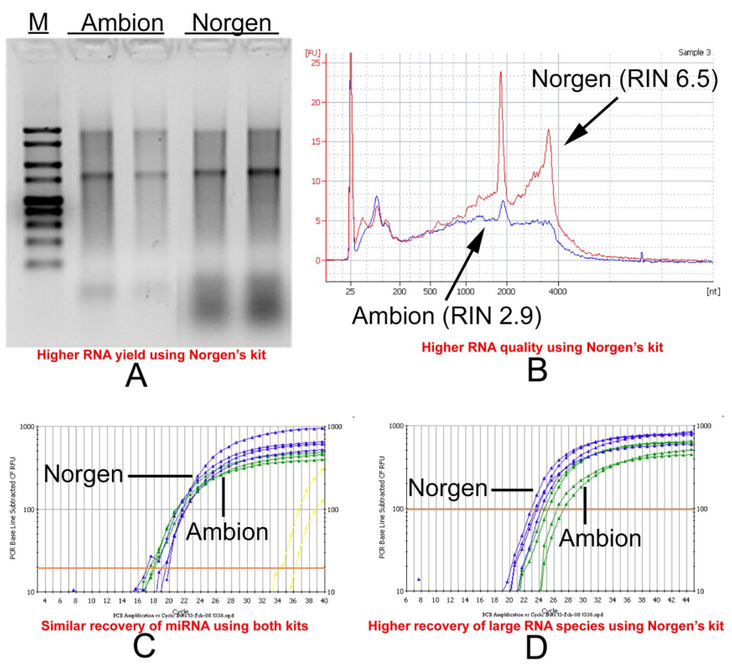

Figure 1. The Difference in FFPE RNA Quality Between Norgen's and Ambion's FFPE RNA Isolation Kits. A) Resolution of FFPE RNA on a 1X MOPS, 1.5% formaldehyde-agarose gel (5µL of the 50µL elutions) for visualization. M= Norgen's 1Kb RNA Ladder. **B)** Total RNA resolved on the Agilent BioAnalyzer. **C)** Detection of microRNA in FFPE kidney total RNA using RT-qPCR with primers specific to miR-21. **D)** RT-qPCR using primers specific for the β-actin gene.

2- Comparison of RNA Yield, Quality, and gDNA Contamination Using Three Systems. Norgen's kit consistently purified total RNA with the highest yield compared to Ambion and Qiagen kits, as displayed in Figure 2A. To determine sample quality, RNA size diversity, and levels of gDNA contamination, delta (Δ) Ct values were used. Δ Ct values were generated from an RT-qPCR using reactions either containing reverse transcriptase (+RT) or not (-RT). Δ Ct assesses the yield and quality of RNA (determined by +RT) and the amount of gDNA in the sample (-RT). Thus a high Δ Ct indicates high quality and yield of RNA as well as a low amount of gDNA contamination. In Figure 2B, Norgen displays higher amounts of miRNA compared to competitor kits, and in Figure 2C, Qiagen and Norgen had similar Δ Ct values for the 5S amplicon, while Ambion had a lower average Δ Ct. The biggest differences could be seen in the large RNA amplicon, β actin (Figure 2D), where Norgen had a higher average Δ Ct as compared with Ambion and Qiagen.

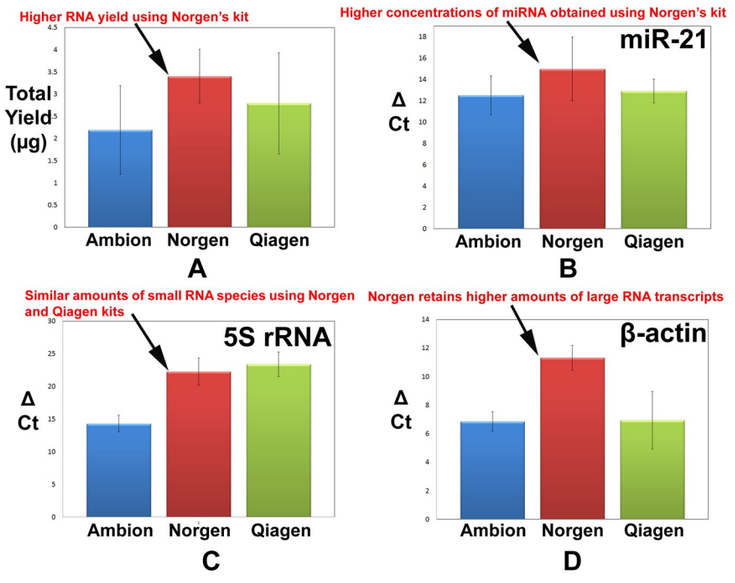

Figure 2. Comparison of FFPE RNA Yield, Quality, and gDNA Contamination Between Ambion, Norgen and Qiagen FFPE Purification Kits. A) Comparison of total RNA yields using 18 sample replicates. **B)** RT-qPCR using miR-21 primers. **C)** RT-qPCR using primers 5s rRNA primers. **D)** RT-qPCR using β-actin primers.

3- Comparison of DNA Yield, Quality and Molecular Weight Range Isolated Using Two Systems. When samples were quantified, Norgen's kit captured much higher amounts of gDNA than Qiagen's kit (Figure 3A). A qPCR was then conducted (Figure 3B), with both kits generating similar Ct values, indicating that both methods elute high quality DNA from FFPE tissues. When the DNA samples were run on an agarose gel, based on the intensity of the bands (Figure 3C), Norgen's kit captured more total DNA, while covering larger molecular weight fragments compared to the DNA isolated by Qiagen's kit. Norgen's kit recovered both very high molecular weight DNA (intact genomic DNA, arrow) as well as small molecular weight DNA. Finally, average 260:280 and 260:230 ratios were used to assess sample quality (Figure 3D). While Norgen and Qiagen samples had similar 260:280 ratios, Norgen's samples had a higher average 260:230 ratio, compared to Qiagen samples.

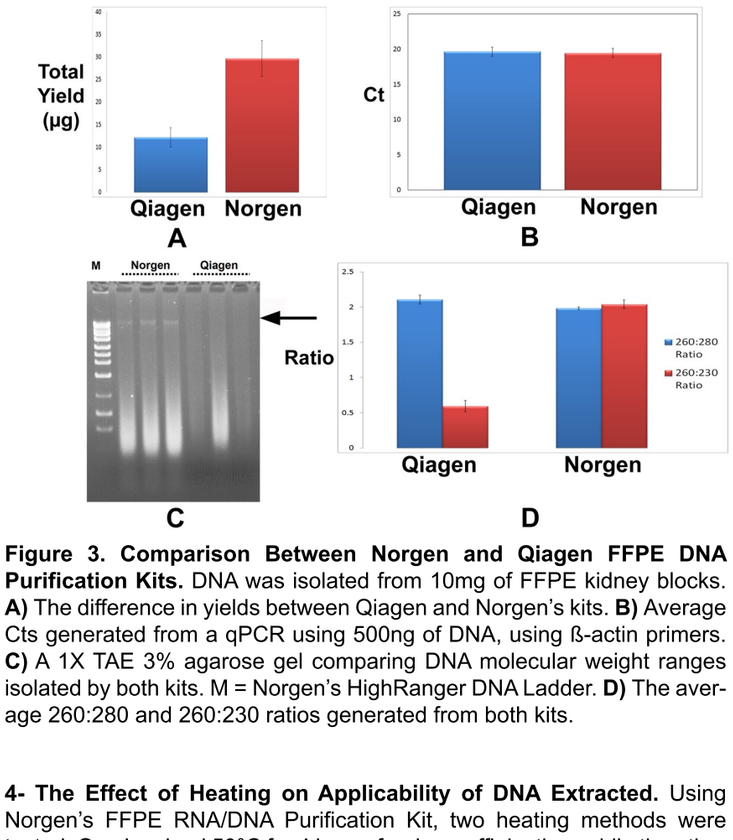

Figure 3. Comparison Between Norgen and Qiagen FFPE DNA Purification Kits. DNA was isolated from 10mg of FFPE kidney blocks. **A)** The difference in yields between Qiagen and Norgen's kits. **B)** Average Cts generated from a qPCR using 500ng of DNA, using ß-actin primers. **C)** A 1X TAE 3% agarose gel comparing DNA molecular weight ranges isolated by both kits. M = Norgen's HighRanger DNA Ladder. **D)** The average 260:280 and 260:230 ratios generated from both kits.

4- The Effect of Heating on Applicability of DNA Extracted. Using Norgen's FFPE RNA/DNA Purification Kit, two heating methods were tested. One involved 50°C for 4 hours for deparaffinization, while the other took place at 50°C for 1 hour, followed by 90°C for 1 hour. Both methods resulted in similar DNA recovery based on the TAE-agarose gel (Figure 4A), with the 90°C heating causing smeary DNA. When 20ng of both samples were used in a qPCR reaction using the ß-actin primer (Figure 4B), 90°C heating resulted in lower Ct values, thus appearing to be the optimal heating method for deparaffinization of FFPE tissues.

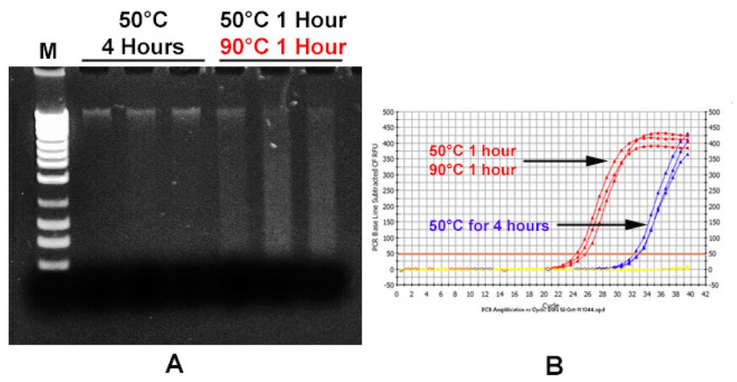

Figure 4. Comparison Between Two Heating Methods for FFPE Deparaffinization. A) 15µL of each method's 150µL elutions were loaded on a 1X TAE 3% agarose DNA gel. M= Norgen's HighRanger DNA marker. **B)** A qPCR reaction of DNA isolated from both heating methods, using the ß-actin primer.

Conclusions

1. **Size Diversity.** Norgen's FFPE RNA Purification kit recovers a greater diversity of RNA fragments due to its unique technology not employed by competitor kits.
2. **Nucleic Acid Quality.** Norgen's kits were found to elute RNA and DNA of higher quality than competitor kits, based on BioAnalyzer results, 260:280 and 260:230 ratios, as well as RT-qPCR and qPCR performance.
3. **Nucleic Acid Quantity.** Norgen's FFPE RNA Purification Kit as well as Norgen's FFPE RNA/DNA Purification Kit consistently isolated higher yields of RNA and DNA than the leading competitor's kits.
4. **Heating During Deparaffinization.** The heating method chosen during deparaffinization greatly affects the quality of DNA isolated from FFPE tissues.

References

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