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Isolation of total nucleic acids from FFPE tissues using FormaPure DNA

Jung Hoon Doh, Ph.D. Senior Application Scientist | Beckman Coulter Life Sciences, Indianapolis, IN USA

Summary

Extensive archives of formalin-fixed, paraffin-embedded (FFPE) tissues combined with next generation sequencing (NGS) capabilities provide rich resources for identifying and validating new biomarkers. However, FFPE preserved samples present challenges for reliable nucleic acid isolations due to extensive protein-nucleic acid and nucleic acid-nucleic acid crosslinks. Also, nucleic acids are heavily fragmented and chemically modified to such a degree that renders them useless in many downstream molecular analyses. Furthermore, degradation of nucleic acids is typically compounded by poor isolation methods that are complex and cumbersome to perform. In this application note, we describe a highly efficient and simple process for isolating both DNA and RNA from FFPE samples using Beckman Coulter's FormaPure DNA kit. Modifications to the original protocol were employed to achieve optimal yield and purity of FFPE derived RNA, and we demonstrate superior RNA functionality via real-time quantitative reverse transcription PCR (qRT-PCR) and next generation sequencing (NGS). Using the following protocol (summarized in Figure 1), nucleic acids from FFPE can be reliably and reproducibly purified allowing for retrospective studies of diseased tissue at both the genomic and transcriptomic levels.

Figure 1. Process overview. Deparaffinization, lysis and decrosslinking incubation times and temperatures were modified to retrieve high quality RNA from FFPE tissues. Total nucleic acids can be isolated by dividing the samples after the lysis step (refer to text for details).

Materials and Methods

Single 10 μm curls of stomach, breast, lung and liver FFPE tissues were used as input samples for all FFPE extractions. Isolations were performed in a nuclease-free environment using 1.5 mL microcentrifuge tubes. All solution volumes used were equivalent to that stated in FormaPure DNA protocol unless otherwise specified. Heated incubations were performed using water baths. For stomach and breast FFPE samples, a Peltier and orbital shaker were used in parallel, in lieu of a water bath and vortexer, to demonstrate automation compatibility with Beckman Coulter's Biomek workstations.

The following modifications were made to the original FormaPure DNA protocol. Deparaffinization was done at 60°C for 10 minutes with brief vortexing every two minutes. Tissue digestion was conducted at 55°C for 60 minutes. It is important to note that minimizing the time of the tissue digestion may increase the recovery of higher quality nucleic acids, especially RNA, and depending on the size and type of the tissue, the full 60 minutes may not be necessary. Decrosslinking was performed at 60°C for 30 minutes for RNA isolations and at 80°C for 60 minutes for DNA isolations. After the decrosslinking step, the bottom layer was transferred to a new tube and the samples were spun at 10,000 × g for 1 minute to pellet cell debris. The clear supernatant containing nucleic acids was processed as follows:

For RNA isolations

The bind step was performed as indicated in the FormaPure DNA protocol. Then, samples were washed once with 400 µL of Wash solution. The 80% ethanol wash was omitted at this stage of the bind-wash-elute process but can be implemented as it may increase purity of the RNA isolates. The samples were eluted with an 80 µL volume of nuclease free water. To the eluate, 10 µL of 10X DNAse Buffer and 10 µL of DNAse I were added (user supplied). The samples were mixed by gently pipetting up and down 10 times and then incubated at 37°C for 20 minutes. After DNAse treatment, 150 µL of Bind solution was added, mixed and incubated at room temperature for no less than 5 minutes. Samples were placed on the magnet for 10 minutes (or until the solution was clear), and the supernatant was aspirated without disrupting the beads. Samples were then washed once with 750 µL of freshly prepared 80% ethanol and air dried to ensure that most of the residual ethanol was evaporated. The RNA was eluted with 40 µL of nuclease free water.

For DNA isolations

RNAse A digestion and the bind-wash-elute steps were conducted according to the FormaPure DNA manufacturer protocol.

### For total nucleic acid isolations

Samples were divided after the tissue digestion step by first pipetting 100 µL of the lysate (bottom layer) into a separate tube and then gently pipetting 150 µL of the mineral oil (top layer) on top of the lysate in the new tube. RNA and DNA isolations can be conducted in parallel as described above. Otherwise, we recommend performing RNA isolations prior to DNA isolations. To note, when splitting samples for total nucleic acid isolations, all volumes must be also be halved after the decrosslinking step in order to maintain proper ratios. For example, 150 µL and 75 uL of Bind solution should be used during the first and second Bind steps, respectively, during RNA isolation. Due to the high efficiency of the extractions, splitting samples to isolate both DNA and RNA should still provide ample amount of material for most downstream applications.

Results

RNA and DNA were successfully isolated from FFPE tissues using the FormaPure DNA kit. We demonstrate that the yields are higher or equivalent to other methods (Figure 2). To examine possible RNAse activity in the FormaPure DNA kit, RNase inhibitor was added to all of the components before usage. We observed no differences in yield with or without RNAse inhibitors, suggesting that the kit was free of RNAse activity. However, we recommend using RNAse inhibitors to safeguard against other sources of RNAse contamination.
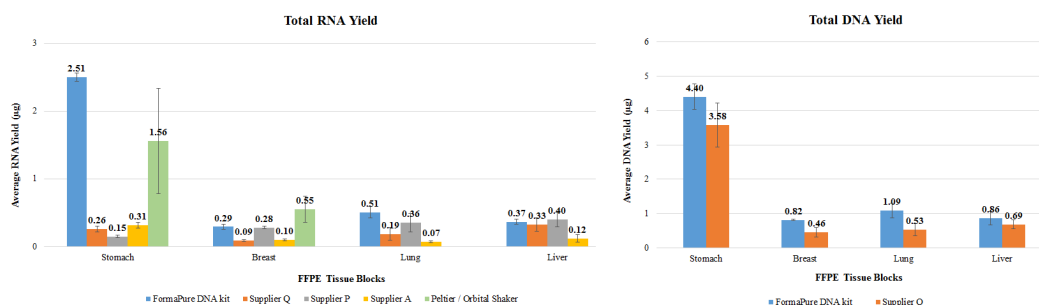
Figure 2. FormaPure DNA recovers higher or equivalent amounts of RNA (left) and DNA (right) from FFPE tissues compared to other methods. Nucleic acids were purified from one 10 µm curl of stomach, breast, lung, and liver FFPE tissues using the modified FormaPure DNA protocol and compared to three different extraction methods. Yield quantifications were performed using the Quant-iT™ RNA and dsDNA Assay Kits (Thermo). The yields represent averages of triplicate isolations.
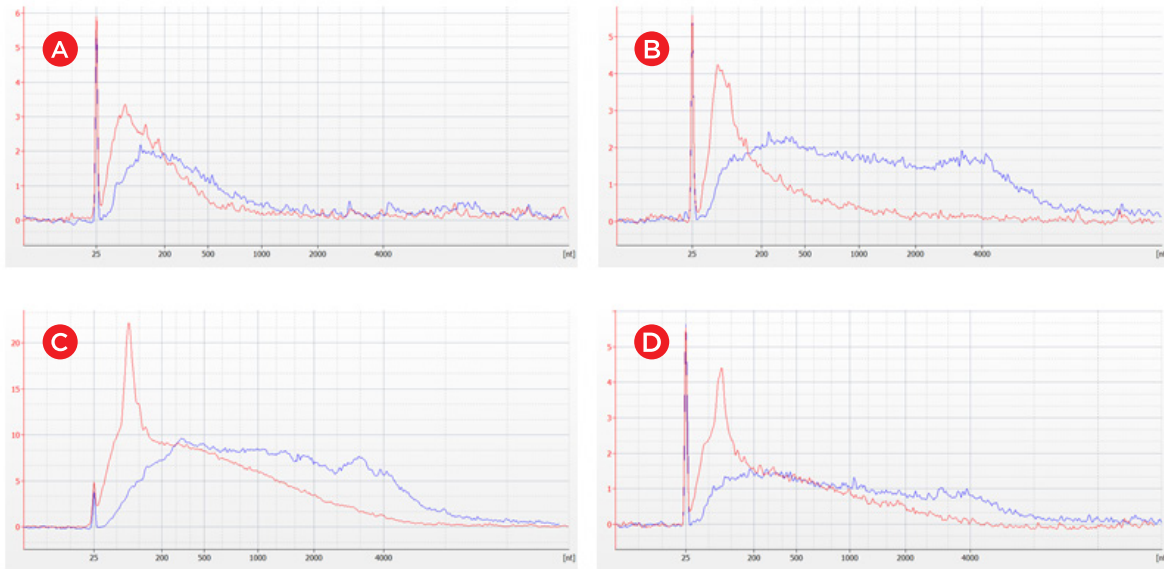
Figure 3. Agilent bioanalyzer traces of RNA isolated from (a) stomach, (b) breast, (c) lung, and (d) liver FFPE samples. Fragment profiles of RNA purified by FormaPure DNA (blue) and supplier Q (red) are shown. Bioanalyzer traces indicate that more intact RNA were purified using FormaPure DNA compared to supplier Q.

We also determined that an onboard Peltier and orbital shaker were suitable substitutes for a water bath and vortexer. RNA isolated from stomach and breast FFPE tissue were higher in yield than other methods when a Peltier and orbital shaker were used (Figure 2), suggesting that FFPE RNA extractions can be automated using Biomek workstations for a higher throughput workflow without compromising the performance and quality.

Cellular RNA is very unstable at high temperatures. In order to preserve the integrity of RNA during the isolation process, lower temperatures and/or shorter incubation times were employed during the deparaffinization, lysis and decrosslinking steps. The intactness of RNA isolated with FormaPure DNA and supplier Q's method were examined via the Agilent Technologies 2100 Bioanalyzer System. Bioanalyzer traces of RNA isolated with FormaPure DNA showed less degraded RNA fragments compared to supplier Q from all four tissue types (Figure 3).

We demonstrate that RNA isolated from FFPE samples using FormaPure DNA are suitable for downstream molecular analyses. First, we assessed amplifiability of RNA via qRT-PCR (Figure 4). A primer set (forward primer 5´-ggacttcgagcaagagatgg-3´ and reverse primer 5´-agcactgtgttggcgtacag-3´) was designed to span Exon 4 and 5 of the beta (β)-actin gene (ActB) to produce 233 and 327 base pair amplicons from cDNA and genomic DNA (gDNA), respectively. This primer set was selected to (1) mimic the size of amplicons that are typically generated for most NGS library preparation protocols and (2) detect gDNA contaminants when qRT-PCR reactions are run without reverse transcriptase (-RT). The FormaPure DNA kit isolated more amplifiable RNA than supplier Q with the same amount of starting material (5ng of RNA). As expected, we observed that more intact RNA gave lower cycle threshold (Ct) values, and vice versa (Figure 3 and 4). Here, RNA isolated from breast, lung and liver FFPE tissues gave average Ct values of 23.9 +/- 0.423, 25.2 +/- 0.252 and 25.5 +/- 0.525, respectively, for FormaPure DNA, which were all lower than those observed with RNA isolated with Supplier Q's method. RNA isolated from stomach FFPE tissue gave overall higher average Ct values as expected: 31.8 +/- 0.570 for FormaPure DNA and 32.4 +/- 0.918 Supplier Q. Genomic DNA contamination was minimal or undetectable from all RNA isolated using both methods (Figure 4).
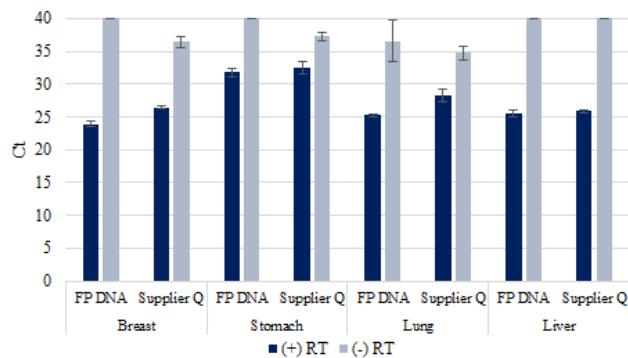
Figure 4. Ct values from real-time RT PCR of RNA isolated with FormaPure DNA vs. Supplier Q. RNA (5 ng) was subjected to one-step real-time PCR with KAPA SYBR FAST One-Step qRT-PCR kit (KAPA Biosystems) using primer sets to the ActB gene. The qRT PCR reactions and its corresponding no RT negative control (no reverse transcription) reactions were performed in triplicates. UD indicates Ct values that were undetermined from a 40 cycle PCR reaction. Refer to text for more details.

Next, to demonstrate NGS compatibility, cDNA libraries of RNA isolated from breast FFPE samples were prepared using the Illumina TruSeq® RNA Access Library Preparation kit. NGS library constructions were performed in triplicates and were accessed following the first and second PCR enrichments using High Sensitivity DNA chips on an Agilent Bioanalyzer (Figure 5). Samples underwent proper PCR amplifications with higher than acceptable yields and appropriate amplicon sizes. The final libraries were > 20nM and at proper fragment lengths. This data suggests that the RNA is compatible with PCR amplifications required in NGS library preparations. NGS libraries were sequenced on the NextSeq™ 500 system, and the sequence reads were aligned using STAR© (Spliced Transcripts Alignment to a Reference) RNA-seq aligner using an Illumina® BaseSpace application (Figure 6).
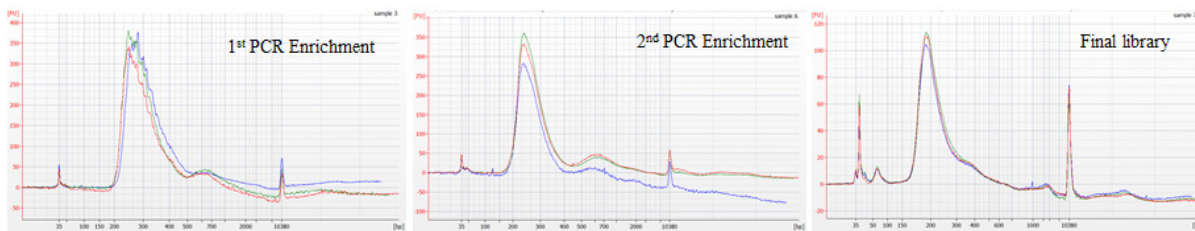
Figure 5. Electrophoretic data of cDNA amplicons, shown in triplicates, following first (left) and second (middle) PCR enrichments, and the final library constructions (right).
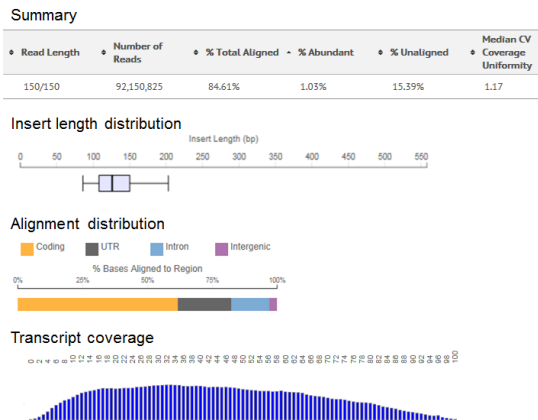
Figure 6. STAR© alignment of sequence reads from FFPE RNA extracted with the FormaPure DNA kit. The sequencing libraries were prepared using the TruSeq RNA Access® Library Prep Kit and sequenced on Illumina® NextSeq™ 500 sequencer.

Conclusion

This study showed that changing the deparaffinization, lysis and decrosslinking incubations is sufficient to recover high quality RNA from a variety of FFPE sample types using the FormaPure DNA kit. The modified procedure can be accomplished in about 3 hours and with less than an hour of hands-on-time. We also demonstrate automation capability on Biomek workstations, which will further reduce hands-on-time time and help significantly increase throughput. Importantly, FormaPure DNA isolated FFPE RNA at higher yields and purities compared to other methods, which led to higher performance in downstream applications.