APPLICATION NOTE

RNA Extraction with EchoLUTION™ from FFPE Tissues Enhances RNA-seq Analysis

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Formalin-fixed paraffin-embedded (FFPE) tissue samples are a valuable resource, providing large and easily accessible collections of cases for genetic studies. Due to chemical modifications and degradation caused by formalin fixation and storage, extracting high-quality nucleic acids from FFPE tissues is challenging, labor intensive, and frequently uses hazardous reagents. To overcome these limitations, we developed a novel method to extract RNA from FFPE tissues to improve convenience and prove compatibility with transcriptomic analysis. The EchoLUTION technology addresses key issues encountered during the purification process. We validated the performance of the technology by RNA extraction from standardized human FFPE samples (tumor-normal pairs) and assessed their quantity and quality. Then we evaluated its compatibility with RNA-seq analysis. The results indicate that rRNA depletion is more efficient for FFPE RNA samples extracted with EchoLUTION than with a conventional silica-based method. In addition, RNA sequencing analysis demonstrated that the number of unique mappers and detected genes were larger with EchoLUTION. In summary, we demonstrate that the EchoLUTION technology offers improved convenience in handling FFPE samples and enhancing RNA-seq outcomes, opening new avenues for research in areas like molecular pathology and personalized medicine.

Introduction

Formalin-fixed paraffin-embedded (FFPE) tissues are a valuable resource not only in disease research, particularly cancer, but also in clinical settings focused on patient care. In oncology, the possibility of capturing the whole tumor transcriptome from these challenging samples has improved diagnosis and facilitated more precise drug selection for personalized treatment strategies. However, the extraction of high-quality RNA from FFPE tissues poses significant challenges due to the chemical modifications and degradation caused by formalin fixation. The quality of RNA recovered from FFPE samples can differ significantly between specimens and even among samples from the same specimen. RNA is often highly fragmented, and gDNA contamination is common during extraction procedures. Therefore, it is essential to select the appropriate RNA extraction kit for FFPE samples. This selection should prioritize a purification method that retains shorter RNA fragments while effectively minimizing the carry-over of gDNA.

BioEcho Life Sciences developed a novel method to extract RNA from FFPE tissues that overcomes critical difficulties during the purification process: the EchoLUTION FFPE RNA Kit. After optimized tissue decrosslinking, and paraffin and detergent removal steps are performed, the RNA is extracted in a single step. The RNA passes through a purification matrix without any interaction thereby avoiding fragmentation bias and the loss of small RNA fragments. Simultaneously, impurities are held back allowing for the collection of

highly pure RNA. Historically, conventional methods for extracting RNA from FFPE tissues have been plagued by lengthy, labor-intensive protocols and the utilization of hazardous reagents. By contrast, the EchoLUTION FFPE RNA Kit workflow offers additional benefits, notably a quicker protocol compared to alternative methods. Moreover, this technology eliminates the need for xylene or other organic solvents, such as ethanol, minimizing the use of hazardous reagents and mitigating safety risks for operators.

We validated the performance of the EchoLUTION FFPE RNA Kit across a diverse selection of standardized human FFPE samples, assessing RNA quantity and quality in comparison to two silica-based extraction kits. Our novel protocol consistently yielded high RNA quantities and integrity, surpassing the performance benchmarks set by the silica-based methods. To evaluate the efficiency of RNA extracted from FFPE tissue using the EchoLUTION FFPE RNA Kit with whole transcriptome RNA sequencing, we selected tumor-normal pairs from brain samples to undergo rRNA depletion followed by library preparation, and subsequent RNA sequencing. Data analysis suggested that rRNA depletion was more efficient for FFPE RNA samples extracted with EchoLUTION than with a conventional silica-based method. rRNA depletion reduces the abundance of redundant rRNA sequences in the sequencing library, allowing for more efficient utilization of sequencing resources and deeper sequencing coverage, and increased accuracy and reliability of the sequencing data. Particularly noteworthy is that the RNA sequencing analysis demonstrated a larger number of unique mapping reads and as a result more detected genes with the assessed method compared to a silica-based purification.

Materials and Methods

RNA extraction from FFPE tissue samples

Reference Medicine, Inc. provided standardized FFPE tissue samples for the study: human tumor-normal colon, brain, kidney, and breast pairs. We cut the FFPE tissue blocks in curls of 10 µm thickness with a Leica® RM2255 Microtome (Leica Biosystems, Germany), and transferred one curl per sample to a tube for the RNA purification. RNA extraction was performed following the manufacturers' instructions. To compare the efficiency of the BioEcho technology with two conventional silica-based RNA extraction methods, we isolated RNA from a series of FFPE samples using the EchoLUTION FFPE RNA Kit (BioEcho, Germany), and two different FFPE RNA isolation kits, referred to here as Kit 1 and Kit 2.

RNA quantification and quality analysis

To determine the quantity and quality of the RNA, we measured the concentration and yield with fluorometry using Qubit™ RNA BR Assay Kit (ThermoFisher Scientific®, USA). Then, we analyzed RNA integrity represented by DV200 (%) using a TapeStation® 4150 System and the RNA ScreenTape® Analysis (Agilent® Technologies, USA).

RNA quality assessment by RT-qPCR

RT-qPCR was used to assess RNA quantity and quality further using an amplified region between the exon 5 – 6 of the DDX5 gene to ensure specific RNA amplification. We executed two different sets of RT-qPCR. The first one included an equal input sample volume (4 µL) from the original RNA extracts, each one with a different concentration. In the second one, we equalized the concentration of the samples obtained with the different extraction methods: 25 ng/µL for lung, 30 ng/µL for breast, 15 ng/µL for kidney, and 20 ng/µL for brain samples. Also, we used 4 µL of sample input, being the total amount of RNA: 100 ng for lung, 120 ng for breast, 60 ng for kidney, and 80 ng for brain. RT-qPCR was performed in a GoTaq® Probe 1-Step RT-qPCR System (Promega®, USA).

Total RNA sequencing

To assess the RNA sequencing compatibility, RNA was isolated from brain tumor-normal FFPE tissue pairs with the EchoLUTION FFPE RNA Kit and with another silica-based extraction kit (Kit 3). We used two FFPE tissue slides (10 µm thickness) per sample, one per reaction tube. 50 µg of total RNA were subjected to rRNA depletion with the RiboCop rRNA Depletion Kit HMR V2 (Lexogen, Austria) following the manufacturer's instructions. Then 1:10 of the depleted material (5 ng total RNA equivalent) was used for library preparation with the CORALL Total RNA-Seq V2 Kit (Lexogen) according to the manufacturer's protocol. Libraries were assessed on a Bioanalyzer® High Sensitivity DNA Analysis (Agilent) and sequencing was conducted on a NextSeq® 2000 instrument (Illumina®, USA) in paired-end mode (read 1 = 71 bp, read 2 = 43 bp). All samples were downsampled to a depth of 2 million total reads and alignment was performed with STAR aligner. For gene counting, we used featureCounts to determine the number of uniquely mapping reads on exons.

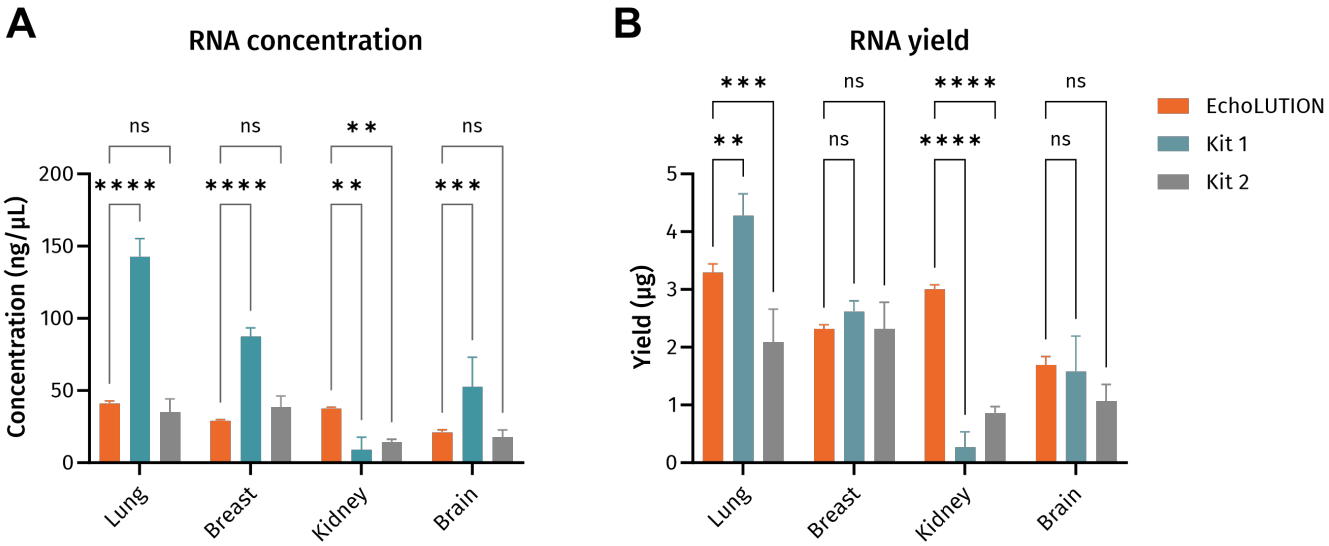

Figure 1. Competitive RNA yield with EchoLUTION FFPE RNA Kit. A. *Concentration of the RNA extracted from FFPE normal samples with three different extraction kits.* ***B.*** *RNA yield data from FFPE tissues were calculated with three different kits. N = 3 replicates/sample. Error bars represent standard deviation. Two-way ANOVA (multiple comparisons); ns (not significant) p ≥ 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.*

Results

The EchoLUTION FFPE RNA Kit provides an efficient workflow with competitive yield and integrity.

To assess the efficiency of the workflows, we evaluated the time each extraction method requires to extract RNA from FFPE tissues using three replicates from each tissue type (lung, breast, kidney, and brain). The EchoLUTION FFPE RNA Kit demonstrated significantly greater efficiency, completing the extraction in just 140 minutes, compared to 205 minutes for Kit 1 and 210 minutes for Kit 2. Then, we estimated the quantity of the extracted RNA for each sample and method (Figure 1). No significant differences existed between the RNA concentration from samples extracted with EchoLUTION and Kit 2 for lung, breast, and brain samples. The concentration of the samples using Kit 1 was, however, higher than the samples from the other two methods (Figure 1A). When comparing the yield of RNA from each of the extraction kits and factoring in the elution volume, these differences disappeared for all the samples except the lung samples which still yielded more RNA from Kit 1 (Figure 1B). The reason for that is that the final output volume with EchoLUTION was higher (80 µL) than the elution volume obtained with Kit 1 and Kit 2 (30 and 60 µL, respectively). For kidney samples, both concentration and yield were higher with EchoLUTION than with the standard methods.

Next, we analyzed the integrity of the RNA (Figure 2). The results revealed that the DV200 percentage was superior for the RNA extracted with EchoLUTION as compared to the RNA extracted with Kit 2 and similar to Kit 1. Again, the EchoLUTION kit outperformed the others with data from the kidney samples with an increased DV200.

Together, these data demonstrate that EchoLUTION FFPE RNA Kit has an efficient workflow that is faster than other kits and delivers high-quality RNA for downstream applications.

Superior amplifiability with the EchoLUTION FFPE RNA Kit

RT-qPCR can be used not only as a technique for transcriptomic analysis but also to qualify RNA for other downstream applications. Any deviation in Ct value may indicate the presence of PCR contaminants (such as DNA) or inhibitors that could affect other applications. As the

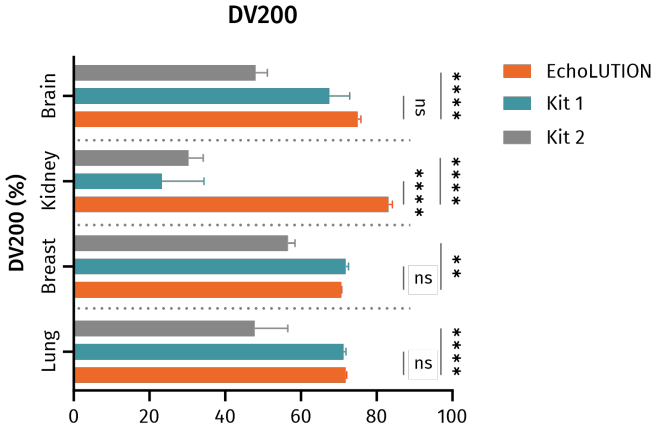

Figure 2. Higher RNA integrity with EchoLUTION FFPE RNA Kit than with other methods. *The proportion of fragments above 200 bp (DV200) is > 70% with EchoLUTION for all samples, similar to Kit 1 (except for the kidney). However, the DV200 proportion is much bigger than with Kit 2. N = 3 replicates/sample. Error bars represent standard deviation. Two-way ANOVA (multiple comparisons); ns (not significant) p ≥ 0.05; ** p < 0.01; **** p < 0.0001.*

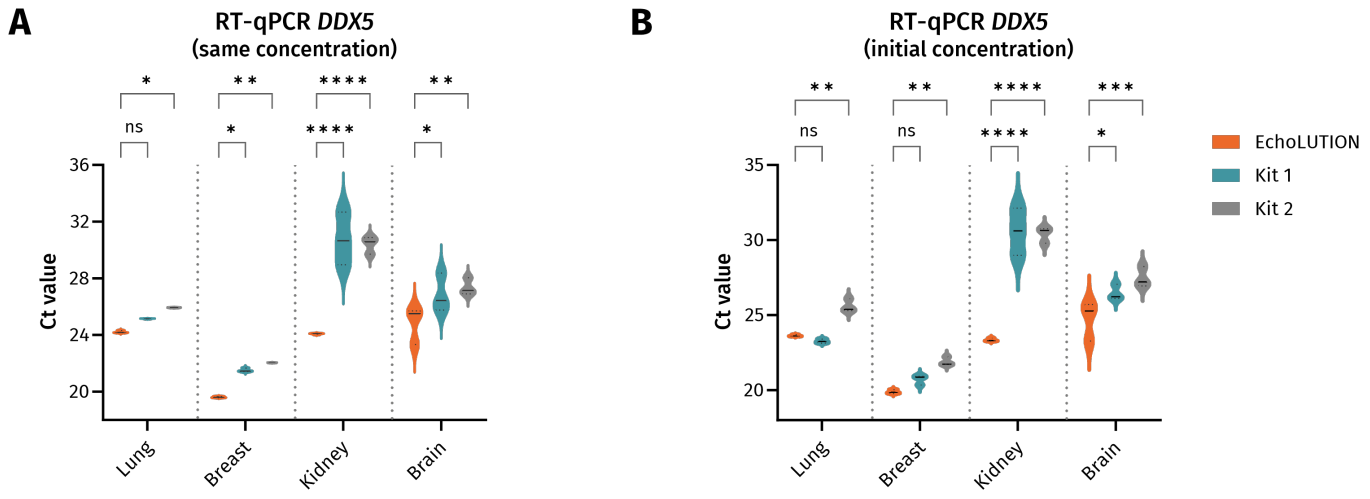

Figure 3. RT-qPCR data demonstrated the high quality of the RNA purified with EchoLUTION. A. *The RNA input amount was equalized for all samples: 100 ng for lung, 120 ng for breast, 60 ng for kidney, and 80 ng for brain.* ***B.*** *The same input volume with the different concentrations obtained after the RNA extraction was used for the amplification of the DDX5 transcript. N = 3 replicates/sample. Error bars represent standard deviation. Two-way ANOVA (multiple comparisons); ns (not significant) p ≥ 0.05; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001*

concentration of the RNA obtained with EchoLUTION was lower for lung, breast, and brain FFPE tissue samples than with Kit 1, we equalized the concentration for all samples to amplify the DDX5 transcript via RT-qPCR (Figure 3A). Results showed that the Ct value for all samples was lower with EchoLUTION than with the other methods, demonstrating that the RNA had the best amplifiability. We also performed RT-qPCR adding the same input volume to the reaction with the initial sample concentrations (Figure 3B). Even with a lower concentration, EchoLUTION exhibited the same or lower Ct values for all samples. This data reveals an optimal performance of the RNA extracted with EchoLUTION, indicating superior purity and quality compared to silica-based methods.

Enhanced rRNA depletion and larger gene counts from total RNA-seq

To determine the compatibility of further downstream applications, we performed RNA sequencing analysis with RNA extracted from FFPE brain tumor-normal pairs using two different methods: the EchoLUTION FFPE RNA Kit and a silica-based kit (Kit 3, here named as silica). Samples were then treated using a set of affinity probes for specific depletion of rRNA. Afterward, the ribo-depleted RNA was used as a template for the library preparation.

Data from the transcriptome analysis demonstrated that the percentage of unique mapping reads after trimming was significantly higher for EchoLUTION samples, in

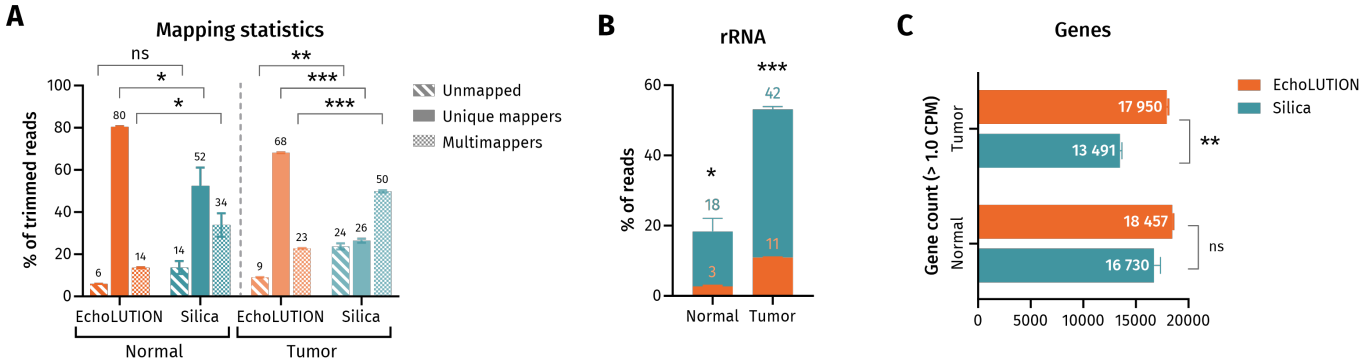

Figure 4. RNA-seq data revealed a larger number of unique mappers and detected genes with EchoLUTION. A. *The percentage of trimmed reads is represented by a total of 2 M reads. The number of unique mapping reads is higher for samples extracted with EchoLUTION than with the silica method.* ***B.*** *EchoLUTION extracted FFPE RNA resulted in significantly fewer rRNA reads in RiboCop rRNA depleted Corall V2 NGS libraries than seen with silica-extracted RNA. N = 2 biological replicates/sample* ***C.*** *The number of detected genes was higher with EchoLUTION than with the silica-based method. N = 2 biological replicates/sample. Error bars represent standard deviation. Multiple unpaired T-test; ns (no significant) p ≥ 0.05; * p < 0.05; ** p < 0.01; *** p < 0.001.*

both normal and tumor brain samples (Figure 4A). The efficiency of the rRNA depletion (Figure 4B) confirmed that the percentage of remaining rRNA was significantly lower in RNA samples obtained with EchoLUTION compared to the samples extracted with the silica-based method. Furthermore, the number of detected genes (> 1.0 counts per million) was larger with EchoLUTION than with the silica-based method: 1,727 genes for the normal brain samples and 4,459 genes for the tumor brain samples (Figure 4C).

These data suggest that, with EchoLUTION FFPE RNA Kit, the transcriptomic analysis is enhanced, providing more data about gene counts and fewer multimapper reads. This underlined the importance of choosing a suitable RNA extraction method, such as EchoLUTION, to get the best information about the transcriptome of FFPE tissue samples.

Discussion

The quality of the RNA isolated from FFPE tissue samples can widely vary depending on the tissue type, the tissue processing (e.g., fixation method, time, and storage), and, of course, the RNA extraction method. RNA derived from FFPE samples generally has relatively low quality and the number of accessible transcripts varies from sample to sample due to cross-linking. Therefore, the appropriate FFPE RNA extraction kit should be used to avoid additional fragmentation and ensure optimal RNA purity. Here, we demonstrated that the EchoLUTION FFPE RNA Kit outperforms silica-based RNA extraction methods:

- The processing time needed for the RNA extraction with EchoLUTION is shorter than with other methods. The user can save more than an hour during the procedure.
- The quantity and integrity of extracted RNA were higher or similar to other suppliers' methods.
- Further, the results from the RT-qPCR revealed that the amplifiability of the RNA is better than the kits based on the silica approach. A lower Ct value indicates that fewer impurities and inhibitors are present in the sample, which demonstrates a higher RNA quality with EchoLUTION than with other silica methods.
- RNA-seq data suggest that the rRNA depletion is more effective on EchoLUTION samples than on the samples obtained with the silica-based kit.
- The RNA analysis revealed that the proportion of unique mapping reads was significantly higher, which correlates with optimal rRNA depletion (it usually results in more unique mappers and fewer multimappers). Most importantly, the number of detected genes was larger in brain samples processed with EchoLUTION than with the silica-based method.

Collectively, these data revealed that the EchoLUTION FFPE RNA Kit offers improved efficiency, safety, and quality while delivering high-quality RNA for downstream applications. Our RNA-seq data points to enhanced transcriptome information from FFPE tissues with EchoLUTION than with a silica-based method. Comprehensive transcriptomic analyses, facilitated by the availability of high-quality RNA from FFPE tissues, open new avenues for research in areas like molecular pathology and personalized medicine.

Acknowledgment

Sequencing experiments were performed by Lexogen NGS Services (Austria).

Ordering information

Product	Reactions	Product no.
EchoLUTION FFPE RNA Kit (10)	10	011-005-010
EchoLUTION FFPE RNA Kit (50)	50	011-005-050
EchoLUTION FFPE RNA Kit (250)	250	011-005-250
BioEcho Cap Puncher*	1 piece	050-001-001

*Optional, for convenient handling of spin columns

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