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Crescent Journal of Medical and Biological Sciences
Vol. 6, No. 3, July 2019, 346–349
eISSN 2148-9696

Validation of Total RNA Spin-column and Non-column Extraction Methods for microRNA Expression Analyses in Formalin-fixed Paraffin-Embedded Liver Samples

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Abstract

Objectives: MicroRNAs are small (19–23nt) noncoding RNAs that adjust gene expression post-transcriptionally. In addition, microRNA (miRNA) profile modification is revealed in various abnormalities including cancer. Formalin-fixed paraffin-embedded (FFPE) material archives are valued for searching human diseases. Therefore, the present study aimed to investigate FFPE samples for miRNA expression, as an alternative analyte for gene expression with the growing therapeutic and diagnostic potential. Further, the validity of RNA isolation kits was evaluated in order to identify a preferred method.

Materials and Methods: The two extracted methods were qualitatively and quantitatively assessed in order to compare the archived specimens between these methods. The total RNA and gene expression were quantified in each method as well.

Results: Based on the results, the level of RNA extracted from FFPE tissues and real-time polymerase chain reaction (RT-PCR) of hsa-miR-623, hsa-miR-515-5pin miRNeasy FFPE (P value <0.047) was significantly higher than that in RNAX Plus ($P<0.084$).

Conclusions: In general, the spin column method for miRNA expression profile had better expression compared to the non-column method. The results further confirmed the validity of FFPE tissues as an appropriate resource for miRNA analyses.

Keywords: Spin column extraction, miRNA, FFPE

Introduction

Formalin-fixed paraffin-embedded (FFPE) materials afford pathologically massive documentation for potential use in biomolecular studies that are universally available. Human tissue samples are fixed in formalin and inserted in paraffin for decades for long-term storage (1,2). Therefore, many institutes have large archives of paraffin-block which provide long-term preserving for neoplasms including rare tumors.

FFPE materials are considered a valuable source of study for analyzing gene expression (3, 4). RNA deformation created by formalin fixation and to amino groups modification for all four RNA bases and over time RNA fragmentation and degradation are the main limitations of such samples to study gene expression (5-7).

The results of several studies showed developments in the isolating process for downstream applications such as PCR made them suitable. This provided an immediate evaluation with clinical-pathological parameters. Therefore, biomarkers can emphasize FFPE usefulness for disease diagnosis and prognosis, as well as potentially new therapeutic targets spatially (8,9), even though most of the easily accessible extraction kits control FFPE tissues (10,11). Accordingly, the current study sought to evaluated FFPE samples for microRNA (miRNA) expression, as an alternative analyte for gene expression with growing therapeutic and diagnostic potential. In addition, the validity of RNA isolation kits was studied, followed by identifying a preferred method.

Materials and Methods

A total of 40 FFPE autopsy blocks were randomly selected including normal liver FFPE tissues from the archives of the Pathology Center of Imam Reza hospital, Tabriz University of Medical Sciences. Each block (5 µm) of histological sections was used according to the miRNeasy FFPE kit and RNAX Plus kit protocols. The sterile condition was used for each block in order to eliminate tissue pollution. Then, the reactions completely were performed in an RNase-free environment. It was aimed to express hsa-miR-623 and hsa-miR-515-5p tumor suppressor genes, the sequences of which are provided in Table 1.

Concisely, the 2 above-mentioned protocols had several steps including FFPE tissue blocks preparation, deparaffinization, proteinase K digestion, DNase

Received 23 November 2018, **Accepted** 15 January 2019, **Available online** 3 February 2019

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Table 1. Oligonucleotide Primer Sequences for Real-Time Polymerase Chain Reaction

Primer	Primer Sequence
hsa-miR-623	CCTTGCAGGGGCTGT GTCCAGTTTTTTTTTTTTTTTACA
hsa-miR-515-5p	GGAGTGCCTTCTTTTGGAG GGTCCAGTTTTTTTTTTTTTTTAACG

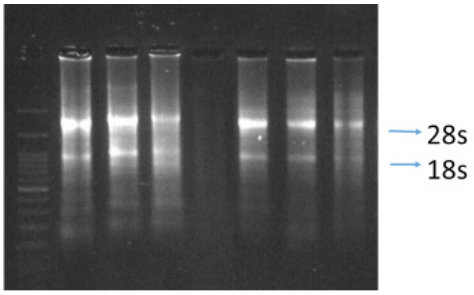

Figure 1. The Comparison of the Integrity of the Total RNA in Both miRNeasy FFPE Kit (left side) and RNAX Plus Kit (right side) Methods.

incubation, and RNA purification. Among the 2 protocols, the miRNeasy FFPE kit was column-based in this study.

For microRNAs Real-time PCR, we used the RealQ Plus ampliqon.

Prime Script RT kit (Takara), was utilized along with random and oligo-dT primers for cDNA synthesis. Prime Script RT kit (Takara) was used either with a primer mix including random and oligo-dT primers, as contained in the kit, or with oligo-dT primer for comparison.

In addition, RNA concentration and quality were measured using NanoDrop and gel electrophoresis, respectively. Further, reverse transcription was performed using the cDNA Synthesis Kit (Takara) from the total RNA, as well as a combination of anchored-oligo (dT) and random hexamer primers (12,13). As shown in Figure 1, high RNA integrity can be observed in the miRNeasy FFPE kit method compared to RNAX Plus method.

Results

The 2 miRNAs expression levels in FFPE were analyzed by comparing both extraction methods. Although the extracted RNA from FFPE blocks is often compromised, in the present study, the robustness of miRNA extraction was demonstrated in miRNAeasy FFPE rather than RNAX Plus kit.

The RNA recovered from FFPE tissues is typically smeared. In higher quality preparations, 2 broad bands representing 18S and 28S rRNA are visible while only a smear is probably observed in most cases.

As shown in Figure 1, the yield of RNAX Plus kit (842±437) is higher compared to miRNeasy FFPE kit (324±108) while the integrity of RNA samples extracted from miRNeasy FFPE kit is clear than that of the RNAX Plus kit (Table 2).

Finally, RT-PCR results of the two genes revealed the efficacy of both methods (Table 3).

Table 2. RNA Yields From FFPE Materials

	NanoDrop Spectrophotometer	
	OD Ratio (260/280)	Yields (ng)
miRNeasy FFPE	1.95±0.12	324±108
RNAX Plus	1.57±0.14	842±437

FFPE, formalin-fixed paraffin-embedded; OD ratio, optical-density ratio. The extraction products were assessed spectrophotometrically using NanoDrop Spectrophotometer and two extractions were conducted using each protocol.

Table 3. RT-PCR Comparison of 2 Genes in 2 Different Methods

RNAX Plus	miRNeasy FFPE	*P* Value
1	1.52	0.047
1	1.34	0.084

RT-PCR, real-time polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded.

Discussion

Archival FFPE samples are regarded as a potential resource for evaluating RNA-based biomarkers in research studies, especially clinical diagnosis. Furthermore, FFPE achieves are universally available and can be validated for clinical case follow up. The RNA extraction from such samples is challenging due to chemical modifications and over time degradation (14).

Doleshal et al discovered that RNA quality of FFPE blocks, for down stream pathways are more strong and have high quality levels from frozen samples (14).

Xi et al showed that the gene expression analyses of the archived FFPE sections between 3 and 20 years of age looked appreciate for high-quality RNA analyses both in clinical prognosis and research studies (9, 15-17).

Kokkat et al studied whether the storage period has noteworthy results on the extracted macromolecules. To this end, they systematically investigated the quality/quantity of genomic DNA, total RNA, and the total protein in FFPE blocks of lung tumors, thyroid, and salivary gland achieved for years and found no significant difference between the macromolecules which were extracted from the blocks stored 2-12 years (18).

Although novel molecular marker profiling has led to an intensive field of research in recent years aiming at diagnosing and treating cancer, only a few markers are introduced into the routine clinical testing.

The recovery of nucleic acids from FFPE human tissues is considered as a growing field in genetic studies. Achieving high-quality methods for recovering high amounts of nucleic acids and yielding amplifiable copies is regarded as a challenging issue for the researchers. In the present research, a test comparison of 2 FFPE extraction

methods was studied on 40 random FFPE liver tissues. The results of the current study showed that miRNA molecules may be effectively extracted and analyzed from FFPE materials.

The total RNA was isolated from 40 FFPE blocks ranging in age from 1 to 4 years and healthy liver tissues and hepatocellular carcinoma were involved to comprehensively assess the two isolation kits. To evaluate the quality and efficiency of miRNA extraction from FFPE tissues and hsa-miR-515-5p genes was studied using ampliqon master mix kit. The average RNA recovery from non-column isolations was higher than that of the spin column yields. However, downstream analyses revealed high-quality results while lower cycle threshold in RT-PCR analyses isolated by RecoverAll by an average of 1.52-fold and 1.34-fold for hsa-miR-623 and hsa-miR-515-5p genes, respectively, was seen (9, 14-17). Actually, the average outcome of miRNA assessment in FFPE blocks in the mini spin column was approximately 2-fold higher compared to the RNAX Plus kit.

Conclusions

In general, the spin column-based extraction method was found to be the preferred method for the recovery of amplifiable RNA copies. Further studies are probably required for specifying the FFPE miRNA expression profiles among different tissue samples.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

This study was approved by Tabriz University of medical Sciences (Ethics No. IR.TBZMED.REC.1396.941).

Financial Support

This study was supported by the grants (60848) from the student research center, Tabriz University of Medical Sciences, Tabriz, Iran.

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