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Comparison and Optimization of RNA Extraction from Formalin-Fixed Paraffin-Embedded Tissues of Hepatocellular Carcinoma

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Abstract

Detection of a new molecular marker for diagnosis and treatment of cancer is a growing field of recent research. The main challenge for molecular investigation is nucleic acid extraction from formalin-fixed, paraffin-embedded tissue (FFPE) of fine-needle aspiration (FNA) samples. In this research, we have compared four different commercially available RNA isolation kits by evaluating the quality and quantity of total RNA. RNA extraction of 10 FNA-FFPE of hepatocellular carcinoma and 10 normal tissue samples were compared and optimized using four commercially available kits: Isol-RNA lysis Reagent (5-PRIME), Cinna Pure RNA kit (SinaClon BioScience), Denazist RNA extraction kit (DENAzist Asia Biotechnology), and RNeasy FFPE Kit (Qiagen) to use in downstream applications. Evaluation of RNA extracting was done by spectrophotometer and electrophoresis. Also, quantitative reverse-transcription PCR was used for assessing the expression of SOX2. RNeasy FFPE Kit had the highest concentration of RNA between the four commercial kits (106.2 ± 17.15) and also, the highest RNA integrity with some modification. The most preferred kit for RNA extraction based on gene amplification was the RNeasy FFPE Kit, which has the lowest CT due to the high quality and integrity of RNA compared to the other three kits with the same modification. Our results suggested that RNeasy FFPE Kit with some modifications in temperature and incubation time was the best kit for RNA extraction from FNA-FFPE issues to a considerable extent with high purity and maintaining the integrity of RNA.

1. Introduction

Detection of new markers using molecular testing for diagnosis and treatment of cancer is an important and growing field of recent research [1, 2]. Nevertheless, fewer molecular markers are used for clinical diagnosis in routine laboratory tests because of the limitations in obtaining intact nucleic acid from some of the samples. Furthermore, using fresh tissue to obtain high-quality nucleic acid is not possible easily in hospitals or laboratories because of the challenge of collecting, preparing, and storing fresh frozen tissue [1]. The main challenge for molecular investigation is the isolation of nucleic acid from specific samples like formalin-fixed, paraffin-embedded (FFPE) tissues, especially from limited tissues such as a fine needle aspiration biopsy (FNA) [3]. The FNA-FFPE tissues provide a wide source of pathological specimens for potential use in molecular studies [3, 4]. Moreover, FNA can be done on both superficial and deep tissue lesions using imaging tools such as computed tomography (CT) scans and magnetic resonance imaging (MRI) [5]. This method is accurate, safe, minimally invasive, and cost-effective [6-8]. Primary hepatocellular carcinoma (HCC) is commonly diagnosed by liver FNA biopsy [9]. The image-guided FNA biopsy of the liver is the best diagnostic choice for patients with hepatic lesions, because of its accuracy and simplicity [10, 11]. The specificity of FNA biopsy of the liver is close to one hundred percent, and the sensitivity is about eighty-five percent [12]. RNA extraction from FNA-FFPE tissues has many challenges, especially while the samples were stored for a long time [13]. The RNA yield extracted from the FNA biopsy is low because of very small amounts of biopsy samples (14). Various studies have estimated that only fifteen percent of FNA biopsy has sufficient RNA for transcriptional analysis [14]. Although formalin is an excellent substance to deactivate nuclease and preserve the integrity of tissue [13], during the paraffin-embedding process (fixation, embedding, etc.), chemical modifications of nucleic acid and formaldehyde cross-links of nucleic acid-protein or protein-protein were occurring. These changes lead to the degradation of nucleic acid, which may affect the quantity and quality of nucleic acid [15, 16]. Because of these irreversible modifications in FFPE tissue and also a very small amount of sample in FNA biopsy, optimization of RNA extraction and obtaining intact RNA from these types of samples is a considerable topic for many researchers [17].

There are several studies in the field of optimization and improvement of the nucleic acid isolation from FFPE tissues [18-21], but only a few studies conducted a careful comparison between commercial kit methods, so scientists have a problem choosing the method for obtaining a high amount of nucleic acid and also acceptable findings for downstream applications [15]. The usual extraction methods cause a significant loss of nucleic acid. For optimization of nucleic acid extraction from FNA-FFPE samples, first, we should be optimized for deparaffinization from the samples, which are usually done with xylene/ethanol washing steps. The next is the standardization of lysis conditions that depend on the type, size, and composition of the tissue sample. Also, the times of lysis incubation and centrifugation are important to avoid tissue debris. For example, increased incubation time or temperatures by adding a further fresh proteinase K is effective in tissue digestion [1, 15].

In this study, we have compared four different commercially available RNA isolation kits by evaluating the quality and quantity of total RNA. We also introduced the best protocol to gain more yield and integrity and minimize the loss of RNA. The purpose of this study is not to change the procedures of these commercial kits but determine and optimize the best procedure with the highest yield of RNA for downstream applications. Whereas our study focuses on FNA tissue of hepatocellular carcinoma (HCC), to compare gene expression of four kits, we assessed SOX_2 mRNA expression, which is related to disease progression and poor survival of HCC [22].

2. Materials and Methods

2.1 *Tissue samples*

In this study, we collected 10 clinical FNA-FFPE blocks of HCC and 10 normal liver tissue during 2020, from Firoozgar teaching hospital, Tehran, Iran. Formalin fixation and embedding were done with 10% formalin at the pathology unit. Five sections of FFPE tissue samples were dissected by pathologists with a 10µm-thick to enrich the tissue content for RNA isolation.

2.2 *Deparaffinization and RNA Isolation*

2.2.1 *Deparaffinization and optimization*

Before RNA isolation from FNA-FFPE tissue, paraffin was removed from the tissues. For deparaffinization, we used xylene and ethanol. First, paraffin is dissolved in 1 ml xylene, and after the precipitation of the tissue, the supernatant is discarded. Residual xylene is removed by washing with ethanol gradient and finally air-dried. After this step, samples were prepared for RNA extraction.

2.2.2 *RNA extraction method*

We compared and optimized four commercial kits for isolating RNA from FNA-FFPE tissue sections; Isol-RNA lysis Reagent (5-PRIME), Cinna Pure RNA kit (SinaClon BioScience), Denazist RNA extraction kit (DENAzist Asia Biotechnology), RNeasy FFPE Kit (Qiagen) (Table 1). We extracted five sections with a 10µm-thick of HCC FNA-FFPE tissue (20 samples for each kit). Generally, we developed the extraction protocols in the commercial kits based on their manufacturers with some modifications due to achieve the best quality. For all kits, the steps of the protocol were the same with an exception in the DNase treatment step that for the RNeasy FFPE Kit was performed during the extraction procedure. For three other kits, DNase treatment was done after extraction by a separate kit before reverse transcription (RT) and qPCR to compare the effects of DNase digestion on the downstream assay. The RNeasy FFPE Kit with longer incubation time, allowing the comparison of different digestion times. We modified some steps of the protocol, such as the incubation time of lysis solution and the amount of proteinase K.

2.3 *Comparison of quantitation and qualification of extracting RNA by four commercial kits*

2.3.1 *Quantitation assay*

As a comparative analysis, we quantitated total RNA in each sample using a nano-spectrophotometer by measuring the absorbance at 260 nm (A_{260}). For calculating RNA concentration, we used the following formula:

Concentration of RNA sample = *44 µg/ml × A_{260} × dilution factor

*44µg/ml is RNA absorbance of 1 unit at 260 nm (A_{260} reading of 1 = 44 µg/ml) at neutral PH

The purity of RNA was evaluated by the ratio between absorbance values at 260 nm and 280 nm (A260/A280). Pure RNA has an A_{260}/A_{280} ratio of 1.9-2.1 in PH 7.5.

2.3.2 *Qualification assay*

The quality and integrity of extracted RNA were assayed by electrophoresis. We run 3µl of RNA on a 1% agarose gel staining with 0.5µg /ml green viewer. For intact RNA, the ribosomal RNA should be appearing as sharp bands, 28S and 18S rRNA with a ratio of 2:1.

2.4 ***cDNA synthesis and qRT-PCR comparison of gene expression***

cDNA synthesis was performed with AccuPower® CycleScript RT Premix (dN_6), K-2046-B, a ready-to-use reverse transcription kit from BIONEER, Korea. For comparing gene expression in four kits, we assessed the expression of SOX_2 and GAPDH as a reference gene by quantitative reverse-transcription PCR (qRT-PCR) using the real-time PCR system (IQ5, Biorad, USA). Primers were designed by Allele ID 7.5 Premier Biosoft and synthesized by Bioneer Company. SOX_2 gene was amplified a 140-bp fragment using SOX_2-Forward (5'-TTGTCGGAGACGGAGAAG-3') and SOX_2-Reverse (5'-GGCAGCGTGTACTTATCC-3') primers. GAPDH gene was used as an endogenous reference gene, amplifying a 126 bp fragment using GAPDH-Forward (5'-GGTGGTCTCCTCTGACTTCAACA-3') and GAPDH-Reverse (5'- GTTGCTGTAGCAAATTCGTTGT-3'), allowing normalization of the expression level of the target gene to the amount of input cDNA. All tests were performed in triplicate for each sample. PCR was performed in a 25 µl reaction containing 10 µl SYBR Premix EX TaqII (2X) (Takara, Cat. #: RR820L, Japan) 0.5-1 µg total RNA input for each sample and 0.05 µM primer sets. Amplification was done under the following conditions: 94°C, 2 minutes, followed by 94°C, 30 seconds; 58°C, 30 seconds, 72°C, 45 seconds and cycled 40 times. A standard curve was obtained from amplification of serial dilutions of pEGFP-C1 vector containing SOX_2 gene (2 to 2 $\times 10^6$ copies). The amplification efficiency and R2 (coefficient of correlation) were 98% and 0.973, respectively.

2.5 ***Statistical analysis***

Statistical analyses were performed using the SPSS version 17.0 software. All data displayed a normal distribution. Quantitative variables were assessed using one-way analysis of variance (ANOVA). Homogeneity of variance calculated by Levene's test. Multivariate logistic regression analysis was used to assess any association in the gene expression, by calculating odds ratios with 95% confidence intervals.

3. Results and Discussion

In our experiments, RNA extraction from 20 FNA-FFPE tissue samples (10 HCC and 10 normal tissue) was compared using four commercially available kits. Table 1 provides a comprehensive overview of all four kits. All samples were analyzed using nano-spectrophotometer and gel-electrophoresis to evaluate yield and quality. Also, for comparing gene expression in four kits, the expression of SOX_2 and GAPDH genes was analyzed at the RNA level with qRT-PCR. The findings are as follows.

Gene expression analysis is a valuable strategy to develop our understanding of the molecular sight and mechanisms of drug resistance in different cancers [23]. Also, the application of molecular biology in the diagnosis and treatment of many diseases, especially cancers, is increasing [24]. Because of the limited amount of FNA-FFPE biopsies, routine tests cannot be done on these samples. Therefore, the use of molecular biology tests such as PCR, real-time PCR, and sequencing are being important for these types of samples [24]. The most important challenges in molecular testing are related to the samples and especially nucleic acid extraction methods. Gaining an appropriate amount of nucleic acid compatible for downstream molecular applications is very important in the

success of molecular tests [25]. Many factors are involved in the quality of the extracted nucleic acids, including the type of sample, sampling and preservation methods, age of the block, and particularly isolation methods [26]. In the current study, by comparing the procedure of four commercially available RNA isolation kits, we have shown that the RNeasy FFPE Kit was the best kit for both quantity and quality in the downstream assay. The yield of RNA obtained from the four kits with different methods and the same modification, from the highest to lowest, was RNeasy FFPE Kit, ISOL RNA lysis reagent, Denazist, and Cinna pure Kit. Assessment of homogeneity of variance using Levene's test for RNA concentration showed that the variance was equal across groups (P= 0.109). ANOVA procedure showed that the highest and lowest RNA concentration between the four commercial kits was RNeasy FFPE Kit (106.2$\pm$17.15) and Cinna pure (30$\pm$12.5), respectively. The homogeneity of variance for optical density (OD)260/280 was equal (P= 0.086). As Table 2 shows, the OD_{260}/OD_{280} ratio of RNA extracted by Cina Pure Kit was the highest (2.404 ± 0.309). The effect size estimated with the partial Eta-squared value for RNA concentration and OD260/280 was 0.112 and 0.334 respectively.

Table1. Comparison of the four protocols of extracting RNA from FNA-FFPE samples

Kit name	**Denazist**	**Cinna Pure RNA**	**Isol-RNA Lysis Reagent**	**RNeasy FFPE**
Purification method	Silica-based membrane	Silica-based membrane	alcohol precipitation	High pure filter
Manufacturer	DENAzist Asia Biotechnology	CinaClon Bioscience	5-PRIME	Qiagen
Catalog number	S-1020	PR891620	2302700	73504
Deparaffinization	Xylene, ethanol	Xylene, ethanol	Xylene, ethanol	Xylene, ethanol
Digestion method	G1digestion Buffer	Lysis solution	Isol-RNA Lysis Reagent	BufferPKD++ Proteinase K
Digestion time	5 min/ RT	5 min/ RT	5 min/ RT	15/56°C & 15 min/80°C
Temperature	(15-25°C)	(15-25°C)	(15-25°C)	
Centrifugation temperature	4 °C	18-25 °C	4 °C	20-25 °C
Sample input	4 x 10 µm Sections	4 x 10 µm Sections	4 x 10 µm Sections	4 x 10 µm Sections
Time required for one sample	1hr & 50 min	15 min	1hr & 20 min	1hr & 30 min
Level of automation	Manual	Manual	Manual	Manual
RNA-binding method	Yes	Yes	No	Yes
Dnase I digest	No	No	No	Yes
Elution volume (µl)	30 µl	30µl	30 µl	30 µl

As we know, the integrity of extracted RNA from these types of samples is compromised and we have not seen distinct 18S and 28S rRNA bands on an electrophoresis gel [27]. Quality analysis by electrophoresis on 1% agarose gel showed that RNA isolated by Denazist kit had the lowest integrity and that the RNA heavily degraded to short fragment lengths (Figure 1), while RNA isolated by RNeasy FFPE Kit (Qiagen) was intact, and the ribosomal RNA, 28S and 18S rRNA with a ratio of 2:1 was appeared (Figure 1).

Table 2. Comparison of four RNA isolation kits based on quantity

Extraction method	OD260/OD280 Mean± SD	OD260/OD230 Mean± SD	RNA Concentration Mean± SD
Isol- RNA lysis	1.630 ± 0.071	0.016 ± 0.005	87.1 ± 4.05
RNeasy FFPE	1.887 ± 0.063	0.033 ± 0.006	106.2 ± 17.15
Denazist	1.447 ± 0.086	0.029 ± 0.011	68.45 ± 27.85
Cina Pure	2.404 ± 0.309	0.016 ± 0.005	30 ± 12.5
P-value	0.03	0.16	0.607

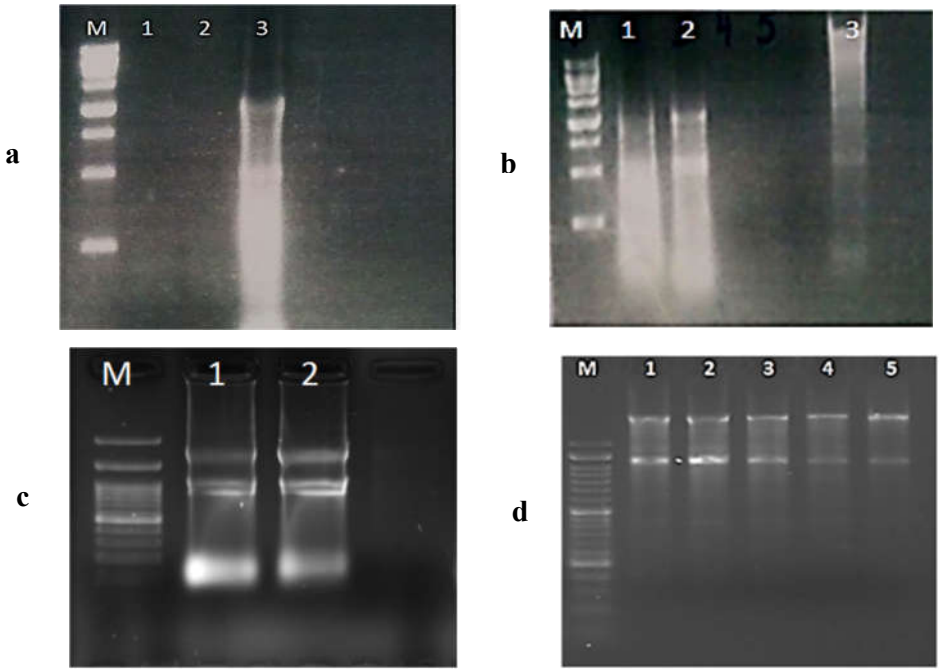

Figure 1. RNA quality analysis in 1% electrophoresis agarose gel by Denazist kit (a), Cinna Pure RNA kit (b), Isol- RNA lysis Reagent (c), RNeasy FFPE (d). RNA isolated from FNA-FFPE tissue (lanes 1-3), Marker 100bp (M)

For evaluating four RNA extraction methods from FNA-FFPE tissues, we select the best kit based on both the highest concentrations of total RNA and the highest efficiency for downstream applications. We found that RNeasy FFPE Kit and Isol-RNA lysis reagent were the two best methods to a considerable extent with high purity and maintaining the integrity of RNA compared to other methods investigated in this study (Table 3).

Table 3. Comparison of four RNA isolation kits based on quantity

Extraction method	OD260/OD280 CV%	OD260/OD230 CV%	RNA Concentration (ng/µl) CV%
Isol- RNA lysis	31	43	4.6
RNeasy FFPE	18	3.3	16
Denazist	38	4.4	40
Cina Pure	31	15	41

The RNeasy FFPE kit is specially designed for the recovery of usable RNA fragments for molecular applications such as RT-PCR. Reversing all mRNA is difficult, because, during fixation and embedding, some RNA will be cross-linked to other molecules. Therefore, a short incubation at high temperatures could be considered as a potential method for reversing nucleic acids caused by formalin [28]. In RNeasy FFPE Kit, there is a heating step at 65 ºC to reverse these cross-links.

One study in 2008 identified significant differences among five commercially available kits [17]. They introduced RNeasy and Recover All, the top two best performing kits, which allowed extracting high yields of RNA with varying sizes, including very small RNA fraction (lower than 100 nucleotides) from FNA-FFPE samples [9]. Variation of kit-to-kit in total RNA yield may be due to the conditions of steps of extraction, including the proteinase K digestion, RNA binding and washing. For example, in the study mentioned above, for binding the sample onto small filter cartridges, they used the highest concentration of ethanol [17]. Furthermore, both protocols recommend identical amounts of proteinase K enzyme and perform this reaction at a similar temperature (200 µg per reaction, 50°C or 55°C for Recover All and RNeasy, respectively).

In our study, longer digestion with Proteinase K yielded more RNA in the RNeasy FFPE kit, while in other kits no difference was observed.

It is also important to note that DNase treatment during extraction will cause loss of RNA to some extent and elimination of this step owing to increase the amount of both RNA and DNA [15]. In the current study, although among the investigated RNA extraction kits, only RNeasy FFPE Kit had DNase treatment step during extraction, it had the best yield of RNA. This may be due to the fact that the DNase by eliminating DNA causes more specific binding of the reverse transcriptase (RT) to RNA by reducing the DNA–RNA hybrids, which might interfere with RT. Furthermore, the efficacy of the extracted RNAs was verified by real time PCR assay. GAPDH mRNA has been commonly used to calibrate genes expression, and the high level of SOX2 gene has been frequently reported in hepatocellular tissues. We compared the four kits for the relative production of RNA for the SOX_2 and GAPDH genes. The gene expression and cycle threshold (CT) in qRT-PCR were compared in the four kits using ANOVA. Levene's test for variance homogeneity showed equality in four kits (P=0.752). The effect size estimated with the partial Eta-squared value was 0.395. There was a decrease in gene expression and an increase in average CT from RNeasy FFPE kit to Denazist kit. In the RNeasy FFPE Kit, the expression of these genes was higher than three other kits, and CT mean was the lowest during q-PCR (Figure 2, Table 4). The gene expression results demonstrated that the most preferred method for RNA extraction based on gene amplification was RNeasy FFPE Kit, which has the lowest CT.

It should be noted that although various results from different kits may be due to isolation methods, sample type, block preparation, or preservation methods, other factors may affect the yield and quality of RNA isolation from these samples. The contamination or defects in steps can greatly reduce the detection of specific RNA targets. In our study, we used positive and negative controls to decrease the influence of work-related factors as much as possible. Generally, RNA isolation from FNA-FFPE tissue is often fragmented and has a lower molecular weight than fresh or frozen samples. In one study in 2010, which compared methods in the recovery of nucleic acids from archival FFPE autopsy tissues, RNA recovered from all samples by 10 extraction methods yielded short RNA fragment sizes of approximately 200 bp or less [15]. As we know, fixation and embedding of tissue influence RNA integrity, but processing before fixation and embedding highly affect the quantity and quality of nucleic acids. There is limited information about how the tissue was processed before fixation and embedding [29]. Several

studies have shown that the ability to assess adequate specimens at the time of biopsy increases overall efficiency if additional tissue is required for further studies [12].

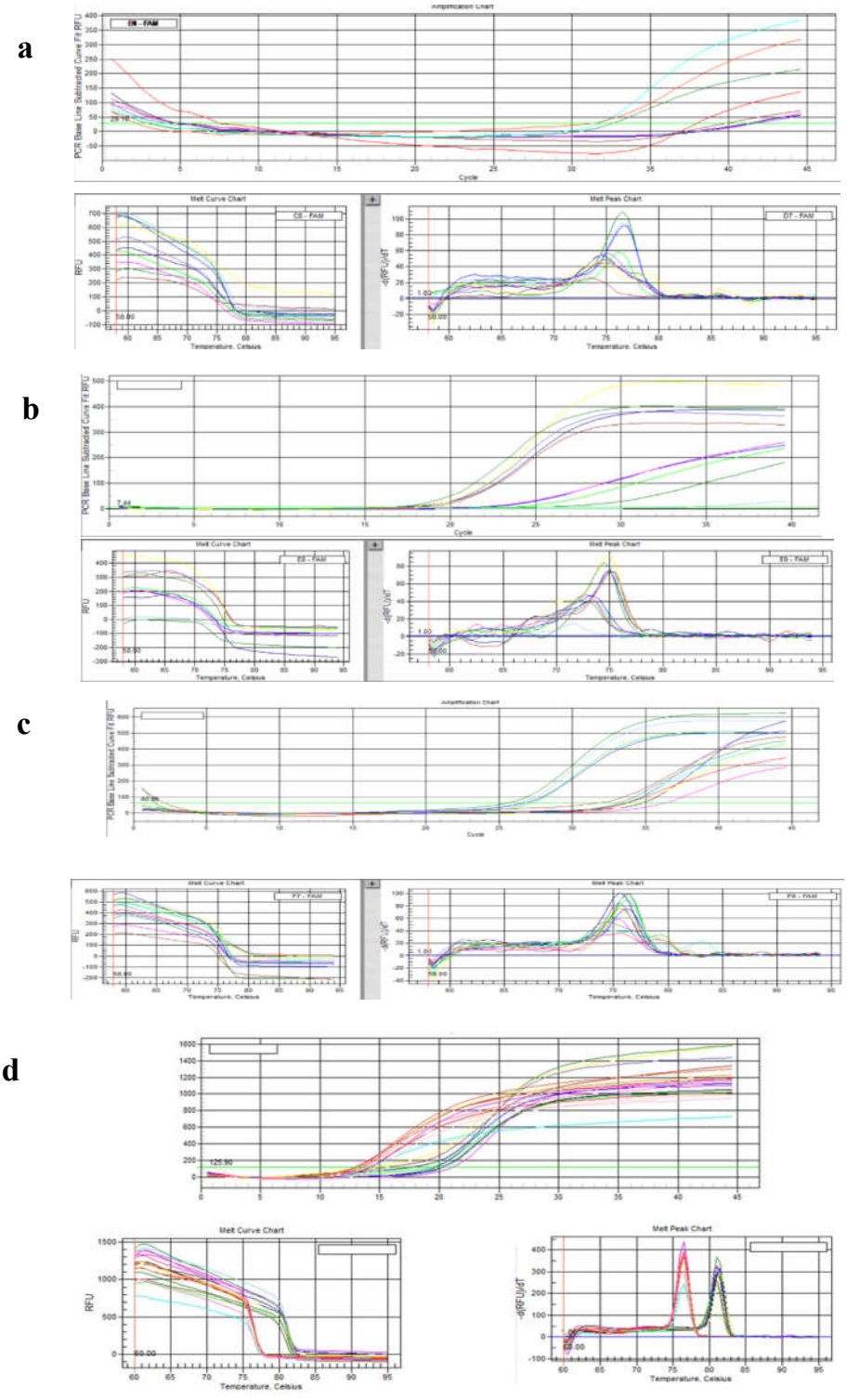

Figure 2. Amplification plot and melt curve of real-time PCR of the SOX2 and GAPDH gene from cDNA of FNA-FFPE tissue RNA extracted by Denazist kit (a), Cinna Pure RNA kit (b), Isol- RNA lysis Reagent (c), RNeasy FFPE (d). RNA extracted by RNeasy FFPE presented lower threshold cycles, most likely due to the higher RNA quality and integrity

Table 4. Comparison of the cycle threshold (CT) of SOX2 and GAPDH genes from RNA samples extracted from the four isolation kits

Extraction method	GAPDH (CT) Mean± SD	SOX2 (CT) Mean± SD
Isol- RNA lysis	25.04 ± 1.39	31.50± 1.11
RNeasy FFPE	13.6 ± 2.06	22.49 ± 1.41
Dena zist	31.6 ± 1.07	36.1 ± 1.67
Cina pure	22.25± 1.28	30.94 ± 1.53
P-value	0.00	0.00

Our study has some limitations. The sample size of this study was small, that may reduce the statistical power of the study and increase the margin of error. Also, the low number of available RNA extraction kits for FFPE tissues was another limitation of this study. Despite the benefits of FFPE tissues, uncertainty about FFPE RNA fidelity remains a serious limitation. FFPE tissue processing and sample storage lead to highly degraded RNA, which may limit gene recognition.

4. Conclusions

Generally, we isolated and analyzed total RNA from 20 FNA-FFPE tissues, including tumor and normal tissue of the liver, using four commercial RNA isolation kits. We successfully isolated total RNA, was consistent with the downstream application. These results should serve as a guide for researchers interested in using these valuable tissues for retrospective molecular studies.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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