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Comparison of RNA isolation from FFPE tissue on two different platforms

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ABSTRACT

Formalin-fixed paraffin embedded (FFPE) tissue is a tissue obtained from dissection of the patients' lesion and is well preserved as a paraffin block. This specimen is regarded as a wealthy resource for RNA studies in various diseases for the advantages of not only diagnosis and prognosis, but also treatment. The aim of our study was to demonstrate that amplifiable RNA could be recovered from FFPE tissues using paramagnetic bead-based isolation technique. RNA isolated on manual and automated platforms was compared for their integrity, yield and purity. Expression of three housekeeping genes from the isolated RNA products was used as a tool to confirm amplificability of the isolated RNA of different amplicon sizes. The results showed that RNA could be successfully recovered from a 10 µm FFPE tissues of 20 samples. The manual platform gave better result for A260/A280 absorbance ratio (1.93±0.009 vs 1.82 ±0.007; p<0.05), whereas the automated platform showed better result with regard to quantity (19.31±2.530 vs 8.87 µg ±1.468 µg). Using reverse transcription polymerase chain reaction (RT-PCR), isolated RNA obtained from both platforms could be achieved with amplicon size up to 656 bp. In this study, we successfully isolated RNA from FFPE tissues using manual and automated platforms under the paramagnetic bead-based scheme with good quality and quantity of the RNA products. Noteworthy, the results also showed longer length of amplified product derived from isolated RNA of FFPE than any other previous reports.

Keywords: Formalin-fixed paraffin embedded, FFPE, RNA isolation, paramagnetic bead-based, manual platform, automated platform

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INTRODUCTION

Low quality of RNA is a problematic feature in downstream RNA-based analysis such as a commonly used method for gene expression analysis, reverse transcription polymerase chain reaction (RT-PCR). Formalin-fixed paraffin embedded (FFPE) tissue, a valuable RNA source, is challenging regarding to RNA degradation, RNA cross-linking with other molecules due to chemical modifications, and limited amount of samples available, which considerably affects its use in downstream applications. Formalin fixation modifies RNA by adding mono-methylol (N–CH_2OH) groups to amino group of all four bases, and also forms methylene bridge between neighboring bases (N-CH_2-N) from amino bases and *N*-methylol condensation, which can interrupt RT. However, chemical modification by formalin can be alleviated under heating step and proteinase K incubation [1]. It should be addressed that fixation process, length of time, condition during sample collection and storage also influence RNA quality [2-4]. The

isolated RNA from FFPE is usually fragmented resulting in size limitation in PCR amplification [5, 6]. Hence, various principles for nucleic acid isolation were developed such as phenol-chloroform method, adsorption method, and density gradient centrifugation method [7]. According to currently available principle, an adsorption method along with magnetic particles was chosen for this study. There are many advantages of using magnetic bead-based separation. Firstly, the particles are possibly separated under magnetic field, thus avoiding a centrifugation step, which often leads to the degradation of nucleic acid. Secondly, commercial kits for numerous sample sources such as tissues, blood samples, cultured cells using the immobilized affinity of magnetic bead to target nucleic acid are accessible. Moreover, due to the easy and achievable magnetic bead-based method, commercial kits are available in both manual and automated platforms [8].

RNA quality is usually defined in terms of RNA purity and RNA integrity, which are accepted as an important concern to ensure reliability and reproducibility of downstream applications. According to a spectrophotometric viewpoint, RNA purity is represented by absorbance ratios of A260/A280, A260/A230, and also A260/A240 [9].The high A260/A280 ratio reflects RNA with little protein contamination. The greater A260/A230 ratio is considered as less residual organic compound contamination such as phenol and alcohol. The A260/A240 ratio less than 1.4 indicates too much salt in sample. However, determination of RNA purity using only the absorbance values and absorbance ratios may not be reliable [10]. These values and ratios provide no information about RNA degradation and sizes of amplifiable amplicons. The 28s/18s ribosomal RNA proportional band is commonly used to define rRNA integrity, thus reflecting mRNA integrity. Though, this approach may be inappropriate for highly degraded RNA from FFPE, which is normally found less than 300 bp in size, and may provide no information on the ability of the isolated RNA to be amplified through RT-PCR. It still remains to be determined whether the extracted RNA can be used as the starting material for the downstream applications [11, 12].

FFPE tissue has been used routinely in clinical diagnosis for decades. We believe that the use of expression profiling based on archival FFPE tissue for identifying new molecular will not only aid diagnosis, but also guide treatment of cancer. For example, estrogen receptor 1 mRNA from FFPE was shown as a prognostic factor in ovarian carcinoma (Darb-Esfahani et al., 2009). Expressions of miRNA in FFPE rectal cancer tissues were found to be predictors of response to neoadjuvant chemoradiation therapy (Kheirelseid et al., 2012). Thymidylate synthase and dihydropyrimidine dehydrogenase mRNA levels on FFPE specimens were considered as predictors for distant recurrence of rectal cancer treated with preoperative chemoradiotherapy (Tanaka et al., 2012). In addition, using FFPE samples, the human epidermal growth factor receptor (HER) family mRNA expression was evaluated for the prognostic ability in breast cancer and for the predictive value with respect to treatment with paclitaxel (Koutras et al., 2008). Therefore, in this study, we tested two platforms of the commercially available RNA extraction kits for their ability to purify amplifiable RNA; the first one was manual platform using FormaPure kit, and another one was automated platform using SPRI-TE FFPE NA extraction kit.

EXPERIMENTAL SECTION

FFPE tissue samples

In this study, 20 different FFPE blocks of breast cancer tissues obtained from patients at King Chulalongkorn Memorial Hospital, who have been previously diagnosed as having breast cancer based on their histopathologic results, were used. The study was approved by the Institutional Review Board (IRB), Faculty of Medicine, Chulalongkorn University (COA No. 111/2012). All samples were collected as anonymous and without clinical data or patient related information. From each sample block, we collected 4 consecutive 10 µm sections. In particular, the first two were used for manual extraction, while the other two were for automated extraction. Since there was one block containing 5 consecutive sections, we had 81 sections totally. Each section was placed into an individual 1.5 ml sterilized microcentrifuge tube, and stored at 4°C until extraction was performed (about 2 weeks).

RNA extraction

We compared 2 platforms of nucleic acid extraction from FFPE tissues (Figure 1); both platforms were solid phase reversible immobilization (SPRI) paramagnetic bead based technology developed by Beckman Coulter, Inc. (Brea, CA, USA). The manual platform was performed using FormaPure Kit (Agencourt Bioscience, Beverly, MA, USA). The extraction processes included tissue digestion and nucleic acid decrosslinking, nucleic acid immobilization with magnetic beads, washing and nucleic acid elution, and final volume was adjusted to 50 µl. The extracted products were stored at -80°C until further analyses. Required chemicals, which were not supplied in the kit, were nuclease-free water (USB, Affymetrix, Cleveland, OH, USA), 100% ultra pure isopropanol (Merck, Germany), 90% isopropanol (freshly prepared with ultra pure isopropanol and nuclease-free water), 70% ethanol (freshly prepared from absolute ethanol (VWR BDH PROLABO, Milan, Italy) and nuclease-free water). Additional device needed was Agencourt SPRIStand - magnetic 6-tube stand for 1.5 mL tubes.

The automated platform was performed on SPRI-TE™ Nucleic Acid Extractor with SPRI-TE FFPE NA Extraction Kit (Nucleic acid extraction from formalin fixed, paraffin embedded tissue). Paraffin melting and digestion steps were manually processed with chemicals provided in the extraction kit, while other steps including binding, washing and elution were performed using SPRI-TE™ Nucleic Acid Extractor. Final volume was set to 50 µl, and the product was kept at -80°C until further analyses.

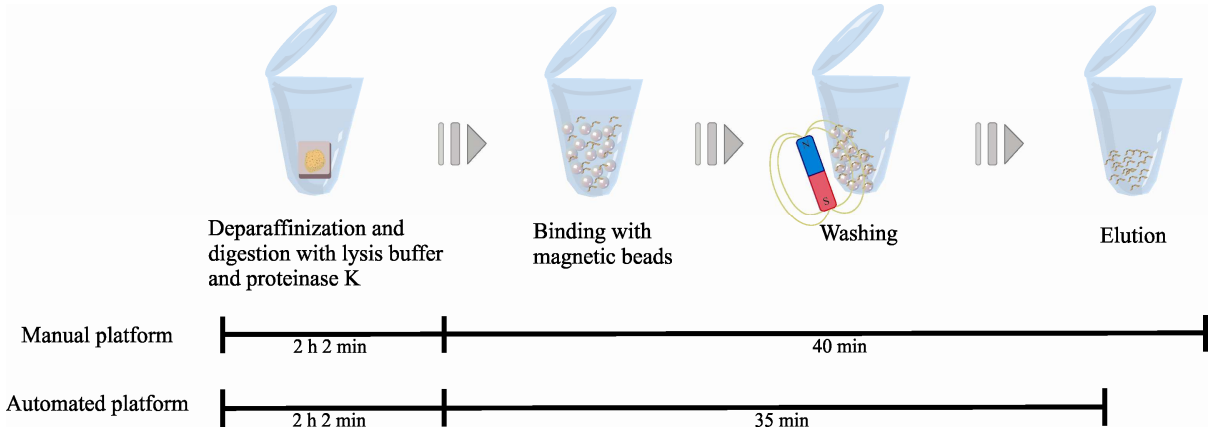

Figure 1. Schematic principle for FFPE extraction using magnetic beads-based method; practical time as stated by manufacturer for a round of manual platform (6 tubes) is 2 h 42 min, and for a round of automated platform (10 tubes) is 2 h 37 min.

RNA measurement

Isolated RNAs were quantified by UV absorption (Thermo Scientific NanoDrop™ 1000 spectrophotometer, Wilmington, DE, USA). The absorbance was measured at 260 nm, 280 nm, 240 nm and 230 nm. Absorbance ratios of A260/A280 and A260/A230 should be very close to 2.00 to determine RNA purity, whereas A260/A240 should be around 1.4 [13].

Recovery of RNA fragments

The isolated RNAs from both platforms (n=5) were randomly selected for electrophoresis on denaturing agarose gel that was 1% agarose gel prepared using formaldehyde denaturing condition with MOPS (morpholinopropanesulphonic acid) buffer, followed by ethidium bromide (EtBr) staining. Samples were loaded onto the gel and electrophoresed. The proportion of 28s rRNA and 18s rRNA bands were observed under UV light of gel documentation system and analyzed by GeneTools software 3.08 (SynGene, Cambridge, UK). The intensity of 28s rRNA nearly 2 times above 18s rRNA was normally accepted as an intact RNA [14].

Table 1. Primer sequences

Name		Sequence	Size (bp)
B2M	Sense	5'-AGGTGACACTATAGAATACAGCCCAAGATAGTTAAGTGGGA-3'	120
	Antisense	5'-GTACGACTCACTATAGGGAAATTCATCCAATCCAAATGCG-3'	
GAPDH	Sense	5'-GACCACAGTCCATGCCATCACT-3'	452
	Antisense	5'-TCCACCACCCTGTTGCTGTAG-3'	
β-actin	Sense	5'-ACGGGTCACCCACACTGTGC-3'	656
	Antisense	5'-CTAGAAGCATTTGCGGTGGACGATG-3'	

Reproducibility of gene expression analysis using RT-PCR method

The similar RNA samples, which were electrophoresed, were reversely transcribed and amplified (n=5). For each 20µl of RT reaction, 5 µl of 100 ng/µl RNA stock was reverse transcribed into cDNA with ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) and 20 U RiboLock™ Ribonuclease Inhibitor (Fermentas, Thermo Fisher Scientific, Ontario, Canada) at 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min to inactivate reverse transcriptase, and then quickly chilled at 4°C according to manufacturer's instruction. Five µl of cDNA product was used for each PCR amplification reaction with recombinant Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific, Ontario, Canada), 0.2 mM dNTP mixture (iNtRON Biotechnology, Korea), 10 µM forward primer, and 10 µM reverse primer (Bio Basic, Ontario, Canada). Three housekeeping genes, B2M, GAPDH [15] and β-actin [16], were used in order to verify RNA integrity. Primers for all genes were designed as intron-spanning to eliminate interference from DNA contamination (Table 1). PCR amplifications were performed according to recommendations (ImProm-II™ Reverse Transcription system, Promega, Madison. WI, USA). Each gene was amplified in separate tubes, the PCR process was initiated with pre-denaturation at 95°C for 5 min, 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C (B2M and GAPDH) for 30 s or 58°C (β-actin) for 30 s, extension at 72°C for 55 s, and post-extension at 72°C for 15 min. PCR products were electrophoresed with TAE

(Tris-acetate-EDTA) buffer on 2% agarose gel for GAPDH and β-actin, and 3% agarose gel for B2M, then visualized by ethidium bromide (EtBr) staining using gel documentation system, and ribosomal integrity was analyzed by GeneTools software 3.08 (SynGene, Cambridge, UK).

STATISTICAL ANALYSIS

SigmaPlot version 10 software (Systat Software, Inc., San Jose, CA, USA) and Microsoft Office Excel 2007 were used for statistical analysis. Means, medians, SDs, SEs and 95%CIs were calculated. Statistical significant difference between manual and automated platform was compared using Student *t*-test ($p < 0.05$).

RESULTS

The assessment of RNA integrity

According to RNA measurement using NanoDrop™ 1000 spectrophotometer, RNA purity and yield under UV absorption were shown in Table 2. Absorbance ratio for 260/280 was significantly different between the two platforms ($p < 0.05$); manual platform gave better result with average ratio 1.93 (±0.009 SEM) vs 1.82 (±0.007 SEM) of the automated platform. However, both platforms provided good result for RNA purity (average ratio greater than 1.8). The automated platform was significantly better than the manual platform in term of quantity ($p < 0.05$). While total RNA obtained from automated platform can be seen up to 19.31 µg (±2.530 SEM), yield from manual method was 8.87 µg (±1.468 SEM).

Table 2. RNA measurement using NanoDrop™ 1000 spectrophotometer

	Method of extraction									
	Manual method (n=20)					Automated method (n=41)				p value
	mean	SD	SE	95%CI		mean	SD	SE	95%CI	
A260/A280	1.93	0.04	0.01	0.02		1.82	0.04	0.01	0.01	0.000*
A260/A230	1.77	0.49	0.11	0.23		1.59	0.35	0.05	0.11	1.22E-01
A260/A240	1.47	0.15	0.03	0.07		1.39	0.19	0.03	0.06	7.95E-02
RNA concentration (ng/µl)	177.41	131.27	29.35	61.44		386.15	323.95	50.59	102.25	7.62E-03*
Amount (µg)	8.87	6.56	1.47	3.07		19.31	16.20	2.53	5.11	7.62E-03*

*Student *t*-test ($p < 0.05$) was used to determine statistically significant difference between two extraction methods.

RNA on denaturing gel was observed in Figure 2a. The proportion of ribosomal RNA (28s/18s) was analyzed, and no significant difference between two platforms was observed ($p>0.05$). An estimated ratio was 1.93 ±1.257 and 1.74 ±0.1116 for manual and automated platforms, respectively (Figure 2b).

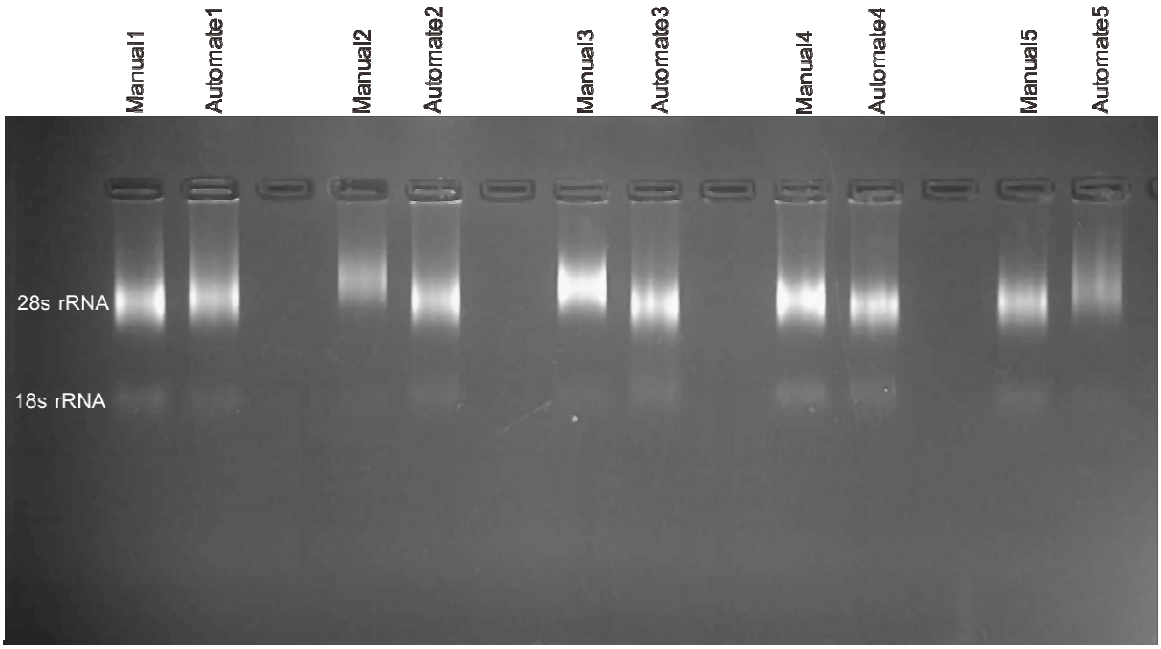

Figure 2a. RNA isolated from both platforms (n=5) were electrophoresed on traditional denaturing agarose gel, and the result was analyzed with GeneTool software. There was no significant difference between the two platforms (p>0.05).

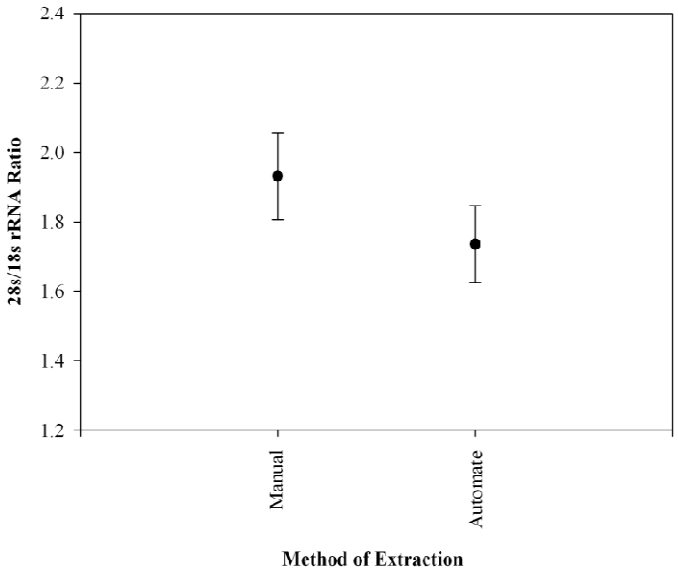

Figure 2b. RNA integrity were expressed as mean ± SE of 28s/18s rRNA ratio which were 1.93 ± 1.257 and 1.74 ± 0.112 for manual and automatic platforms respectively.

RNA reproducibility

The reversely transcribed RNA was amplified during PCR process; B2M (Figure 3a), GAPDH (Figure 3b) and β-actin (Figure 3c) were chosen for different product sizes as targets. None of them was expressed significantly different between 2 platforms ($p<0.05$, $n=5$). However, not only the amplifiable products from RNA were found, but the products from genomic DNA were also observed. As seen in Figure 3b, the genomic DNA amplicons were created at the length of 557 bp.

DISCUSSION

Total RNAs were isolated from 61 FFPE sections using manual (FormaPure kit) and automated platforms (SPRI-TE^{TM} Nucleic Acid Extractor and SPRI-TE FFPE NA extraction kit) with the similar principle. Analytical and performance characteristics of the two platforms were compared including purity of isolated RNA, RNA integrity, RNA reproducibility and practicability.

For the manual platform, magnetic field is formed under magnetic separators, which are designed to held tubes of various sizes. The 6-tubes stand is designed for 1.5-1.7 ml tubes, while the 96-wells stand is designed for 0.2 ml microtubes. A variety of designed magnetic separators are appropriate to separate magnetic particles from different ranges of volume. The magnetic field for automated platform referred to SPRI-TE^{TM} Nucleic Acid Extractor is formed under magnetic bar, and the holders are restricted to 2 ml tubes (available in commercial kit). RNA isolation can be performed in10 tubes at the same time with the automated platform. Paraffin melting, nucleic acid de-crosslinking and tissue digestion steps are needed to perform manually in both platforms. Time spent for these steps is about 2 h 2 min. Time for binding, washing, separation and elution processes are about 40 min for 6 tubes under manual platform and around 35 min for 10 tubes using automated platform (Figure 1). As a result, both platforms are useful. The drawback of the automated platform is a costly machine, while the manual platform needs only simple apparatus such as water bath or heat block. It is of course time-consuming and laborious with respect to the manual one.

Absorbance ratios were given as mean±SE. For manual platform, A260/A280, A260/A230, and A260/A240 were 1.93±0.01, 1.77±0.11 and 1.47±0.03, respectively as compared with those from the automated platform 1.82±0.01, 1.59±0.05 and 1.39±0.03, respectively. RNA concentration from manual platform was lower than those of the automated platform 177.41±29.35 vs 386.15±50.59 ng/µl, as well the total amount of the isolated RNAs, which were 8.87±1.47 vs 19.31±2.53 µg, respectively. However, the purity of RNA as seen from A260/A280 ratio of the manual platform was significantly better than that of the automated platform.

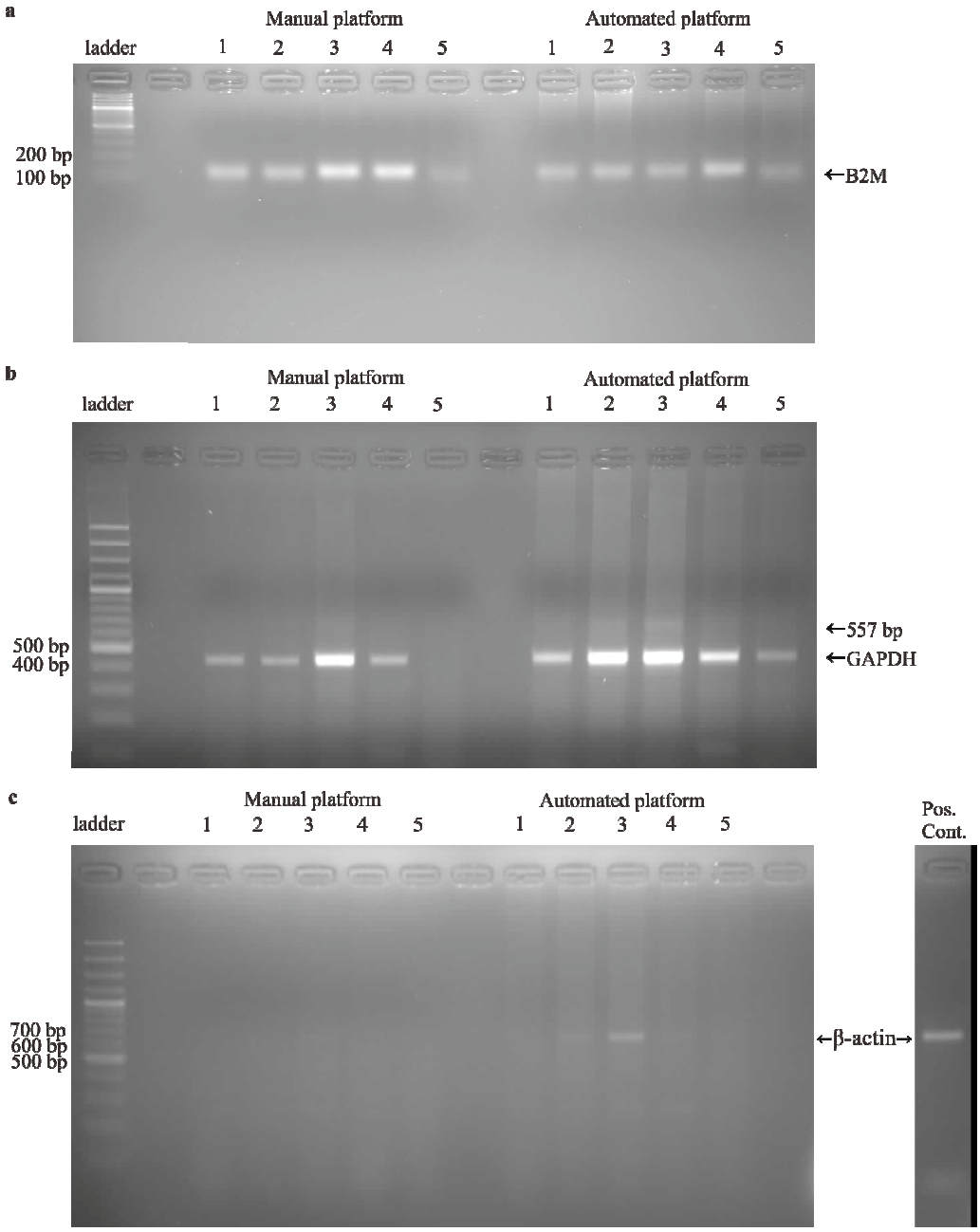

Figure 3. The PCR products were visualized by EtBr staining under gel documentation.
a. An expected product size of B2M from cDNA was 120 bp, and b. an expected product size of GAPDH was 452 bp, both of them were found at the expected size. c. However, β-actin was seen at the expected size of 656 bp in some samples.

The 28s/18s ribosomal RNA ratios were 1.93±1.257 and 1.74±0.112 for manual and automated platform, respectively, which was not significantly different ($p>0.05$), although it seemed to be higher in the manual platform. RT-PCR is used to demonstrate RNA-based downstream application. Three housekeeping genes: B2M, GAPDH and β-actin were tested, and intron-spaning primers were used to minimize genomic DNA interference. An expected amplicon sizes for B2M, GAPDH and β-actin were 120, 452, and 656 bp, respectively. All of them were successfully reversely transcribed and amplified by PCR (Figure 2a and 2b). Nevertheless, the length of 656-bp for β-actin was hardly seen in certain samples (Figure 3). The size of amplicons from this study seemed to be better than an average length of amplicons ever reported in previous works in which RNA recovered from FFPE tissues were around 200-300 bp [2, 17-19].

Though, the RNAs isolated from manual and automated platforms were well amplified at the expected sizes, the contaminated genomic DNA could also be seen at the amplified length of 557 bp in Figure 2b. None of them were shown significantly difference between manual and automated platforms ($p<0.05$). The RNAs isolated from the

manual platform seemed to be better at the amplified short length (B2M), whereas, at amplified long length (GAPDH, and β-actin) were slightly better in RNAs isolated from the automated platform (data not shown).

In summary, RNA isolation from FFPE tissues is normally encountered with recovery and amplification ability. In this study, total RNA can be recovered from all FFPE samples via manual and automated platforms. Both platforms had given appropriate RNA in terms of yield, integrity, reproducibility and longer RNA fragments, thus serving as an alternative technique for RNA isolation.

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