IMPROVING SILICA-BASED SPIN-COLUMN EFFICIENCY FOR RNA ISOLATION FROM FFPE TISSUE

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ABSTRACT

RNA isolation from formalin-fixed paraffin embedded tissue has been a target for improvement for many years. The major limitation is the highly degraded RNA, which in turn increases the demand of the highest yields possible during isolation procedures. In this study, we compared the purification efficacy of RNA between the traditional ethanol precipitation and a modified protocol, which includes the use of silica-based spin-columns for RNA purification and recovery. We also modified and assessed the efficacy of a protocol for effective decontamination and regeneration of spin-columns.

Keywords: *RNA extraction, RNA purification, ethanol precipitation, silica-based spin-columns*

INTRODUCTION

For many decades formalin fixation of tissue followed by embedding in paraffin (FFPE) has been the method of choice to preserve tissue morphology, thus allowing histological study of biopsy specimen for assisting in the establishment of clinical diagnosis (1). With the development of molecular biology analytical methods, a need to isolate genetic material for molecular diagnostics has emerged (2). Many studies on characterization and quantification of RNA molecules have been performed mainly by utilizing different kits for RNA extraction (3–5). Although DNA appears to be quite stable, the major disadvantage of the FFPE tissue preservation is the highly degraded RNA molecules obtained during isolation procedures (6). After extraction of RNA with the assistance of a proteinase K lysis buffer or phenol-based reagents, quite an effective way to overcome this obstacle is by including silica-based spin columns during the isolation procedure (7). Silica-based spin columns are proved to recover high yields of extracted nucleic acid even from tough-to-lyse tissues compared to ethanol precipitation (8). In order to be reused, silica-based spin-columns can be decontaminated by alkaline treatment with the addition of non-ionic detergent Triton X-100 and regenerated by acidic treatment (pH=4) by utilizing a sodium acetate/acetic acid buffer (9).

The aim of this study was to compare and prove the superiority of silica-based spin column RNA purification, as well as the efficacy of decontamination and regeneration of used columns with concomitant

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use of lab-made RNA wash buffers for use with the columns. In this study, we have validated a modified protocol for the effective decontamination and regeneration of used columns and the efficiency of additional RNA purification performed with used columns.

MATERIALS AND METHODS

RNA extraction

Six FFPE tissue blocks were taken to retrieve tissue. Five tissue sections of 10μm thickness were obtained from each FFPE tissue block and were placed in 1.5mL microcentrifuge tubes. The procedure was repeated once more until a final number of 12 sample tubes were obtained. Tissue samples were deparaffinized by adding 1mL xylene to each, followed by a brief vortex and spin at 13.000rpm for 2 min at room temperature. Xylene was then removed by pipetting. The pelleted tissue was then washed from residual xylene by adding 1ml of absolute ethanol. Samples were briefly mixed by vortex followed by spin for 2 min at room temperature. Deparaffinized tissue was left to air dry with open tubes. Then, 100μL of Quickextract FFPE RNA extraction Lysis buffer (Epicentre, Illumina, USA) were added to the tissue sample. Samples were incubated at 56°C for 30 min and further heated to 80°C for 10 min. Then, 1mL of Accuzol (Bioneer, Republic of Korea) solution was added. Samples were mixed by vortex and incubated at room temperature for 8 min. Then 200μL of chloroform were added. Samples were mixed by vortex and allowed to sit for 2 min and centrifuged for phase separation at 10.000rpm at 4°C for 15 min. The aqueous phase was transferred to a new clear 1.5mL microcentrifuge tube.

RNA purification and recovery

Ethanol precipitation

Six samples were purified with the use of ethanol precipitation. An equal volume of isopropanol was added to the aqueous phase and mixed by vortex. The samples were incubated at -20°C for 1 hour. Then, samples were centrifuged at 10.000rpm for 15min at 4°C. The nucleic acids formed a pellet at the bottom. The supernatant was discarded taking care not to dislodge the pellet and 1mL of 75% ethanol was added to each sample and mixed by vortex. Samples were centrifuged at 10.000rpm for 10 min at 4°C. The supernatant was discarded and samples were left to dry. Finally, the pelleted RNA was resuspended in 15μL RNAse/DNAse free water.

Silica-based spin-columns

The RNA from the same tissue samples was purified with the use of silica-based spin-columns. An equal volume of ethanol was added to the aqueous phase and mixed by vortex. The samples were transferred to mini silica-based spin-columns (Zymoresearch, USA) and centrifuged at 10.000rpm for 1min. The flow-through was discarded. For the purification of RNA two buffers were created. The first buffer (A) was composed of 99% absolute ethanol and 1% 3M sodium acetate pH=6.7. The second (B) was composed of 80% absolute ethanol, 19% HPLC grade H_2O, and 1% TAE buffer pH=8.0 (Applichem, Germany). Initially 400μL of buffer A was added to the column and centrifuged at 10.000rpm for 1 min. The flow-through was discarded. Then, 400μL of buffer B was added to the column and centrifuged at 10.000 rpm for 1 min. The flow-through was discarded. The same procedure was repeated with 700μL of buffer B. Columns were transferred to new clear 1.5ml microcentrifuge tube and loaded with 15μL of molecular biology grade H_2O. Columns were left to rehydrate for 5 min and then centrifuged at 13.000rpm for 2 min to recover the RNA.

Column decontamination and regeneration

Two solutions were needed, one for purification and decontamination and one for purification. The first solution (A) consists of 0.2M NaOH and 0.1% v/v Triton X-100 and the second (B) consists of 50mM sodium acetate/acetic acid pH=4.0 (9). First, 500μL of pre-warmed (75°C) solution (A) was transferred to the column and gently pipetted paying attention to avoid agitation of the silica membrane with the pipet tip. Then the solution was removed by aspiration with the pipette in order to remove dissolved high molecular weight proteins or crude tissue particles, which cannot pass through the membrane. Additional 500 μL of pre-warmed (75°C) solution (A) was transferred to the column and was left for 10 min. Columns were then centrifuged at 10.000 rpm for 1 min and the flow-through was discarded. This procedure was repeated once more. Then 500μL of solution (B) were transferred to the columns and incu-

bated for 5 min. This step was followed by centrifugation at 10.000 rpm for 1 min and the flow-through was discarded. Lastly, 700μL of HPLC grade water were transferred to the columns followed by centrifugation at 10.000 rpm for 1 min and the flow-through was discarded. Columns were stored at 4°C. Three decontaminated and regenerated silica-based spin columns were placed in a sterile 1.5mL microcentrifuge tube and then 15μL of HPLC grade water was transferred to the columns. The columns were centrifuged at 13000 rpm for 1 min. The collected flow-through was measured through spectrophotometry.

Six RNA samples were in turn purified with the use of decontaminated and regenerated columns and additionally with the use of new silica-based spin columns. For both groups the purification process was performed with lab-made RNA wash buffers, and the procedure followed as previously mentioned.

RNA quantification through spectrophotometry

RNA sample yield and purity was calculated by spectrophotometry measuring absorptions at 260, 280 and 320nm using Take3 plate of Synergy 2 instrument (Biotek, USA). At 260 nm wavelength nucleic acids readily absorb the emitted light energy and at 280 nm the proteins. The 320nm absorption value is measured as an internal blank.

Statistical analysis

All data presented have been processed using GraphPad Prism V6 and Microsoft Excel 2013 software. For the estimation of statistical significance, students *t*-test were performed with $p<0.05$ considered as significant.

RESULTS

RNA purification efficacy

RNA yields were highly variable and dependent on initial tissue quantity, which underwent extraction. Six identical tissue samples were purified by ethanol precipitation and silica-based spin-columns. The purification efficacy was estimated from the yield and purity of extracted and purified RNAs (Table 1). The concentration of the purified RNA through ethanol precipitation ranged from 226.28ng/μL to 495.14ng/μL, while the silica-based spin column purified samples ranged from 118.24ng/μL to 2084.5ng/μL. The purity of the samples measured by $A_{260/280}$ resulted to an average of 1.752±0.089 for the ethanol precipitation and an average of 1.94±0.095 for the silica-based spin column purification procedure.

*Table 1. RNA yield and purity obtained through ethanol precipitation and silica-based spin-column purification. *p<0.05 ethanol precipitation vs. silica based spin-column*

	Ethanol precipitation		Silica-based spin-column	
Sample	RNA Concentration [ng/μL]	$A_{260/280}$	RNA Concentration [ng/μL]	$A_{260/280}$
1	495.14	1.776	2084.5	1.898
2	399.3	1.762	177.12	1.922
3	226.28	1.611	2012.4	1.99
4	262.84	1.821	118.24	2.025
5	243.8	1.855	2021.04	1.781
6	585.24	1.689	929.28	2.029
Mean RNA purity ± SD		1.752 ± 0.089	-	1.940 ± 0.095
p value of $A_{260/280}$		-	-	0.037*

Decontamination efficacy

The efficacy of decontamination of silica-based spin columns was estimated by measuring the absorption at 260 and 280 nm of the elution volume (Table 2). The values of first sample were A_{260}=0.005 and A_{260}=0.002 and for the rest two samples A_{260}=-0.001, A_{280}=-0.001 and A_{260}=-0.002, A_{280}=-0.001, respectively. The A values below 0.01 are considered extremely low for the first sample and for the remaining two, they were negative.

Table 2. Silica-based spin-column decontamination efficacy

Column	RNA concentration [ng/μL]	A_{260}	A_{280}	$A_{260/280}$
1	3.82	0.005	0.002	2.076
2	-0.42	-0.001	-0.001	0.404
3	1.34	-0.002	-0.001	2.393

Comparison of RNA purification efficacy between new and decontaminated- regenerated silica-based spin columns.

Six RNA tissue samples were purified by new and decontaminated-regenerated silica-based spin-columns. The purification efficacy was estimated from the yield and purity of extracted and purified RNAs (Table 3). The concentration of the purified RNA with the use of new silica-based spin-columns ranged from 375.58ng/μL to 909.58ng/μL, while the respective concentration of RNA obtained with decontaminated-regenerated silica-based spin columns ranged from 299.62ng/μL to 828.16ng/μL. The purity of the samples measured by $A_{260/280}$ resulted in an average of 2.031±0.039 for the new silica-based spin-columns and an average of 2.004±0.048 for the decontaminated-regenerated silica-based spin column purification procedure.

concentration. RNA purified by silica-based spin-columns showed higher purity according to the average of absorption ratio $A_{260/280}$ of 1.94±0.095 compared to the average of absorption ratio $A_{260/280}$ of 1.752±0.089 of the purified RNA samples with ethanol precipitation. Values above 1.8 show an RNA sample with high purity, relatively free from contaminants. The general superiority of silica-based spin-columns against ethanol precipitation can be observed through the higher absorption values at 260nm (Table 1), which gives the concentration value. Additionally, the average absorption ratio $A_{260/280}$ for the purified RNA with the use of silica-based spin columns was significantly higher (p=0.037) than the ethanol precipitation (Table 1). Moreover, the high amounts of RNA recovered show the effective binding of RNA molecules to the silica membrane thus preventing the loss of RNA during washing cycles.

Furthermore, the modified protocol for regenerating columns provides efficient decontamination. The A_{260} and A_{280} values show that the columns were free from nucleic acids and other contaminants, namely proteins, and thus allow them to be reused for purification of further RNA samples (Table 2).

By comparing the new silica-based spin columns with the decontaminated-regenerated columns, we conclude that the purification efficacy is highly similar as estimated by the absorption ratio $A_{260/280,}$ since the purity shows no statistically significant difference between the two variables (Table 3). We did not use RNA concentration to conclude

Table 3. RNA yield and purity obtained through new silica-based spin-column and decontaminated-regenerated column purification

	New Silica-based spin-column		Decontaminated-regenerated Silica-based spin-column	
Sample	RNA Concentration [ng/μL]	$A_{260/280}$	RNA Concentration [ng/μL]	$A_{260/280}$
1	835.18	2.049	436.5	1.982
2	375.58	1.996	299.62	1.944
3	436.38	1.969	440.48	2.009
4	821.74	2.067	740.88	2.073
5	909.58	2.044	828.16	2.042
6	771.74	2.059	355.6	1.973
Mean RNA purity ± SD		2.031 ± 0.039	-	2.004 ± 0.048
p value $A_{260/280}$		-	-	0.235 N/S

DISCUSSION

Silica-based spin-columns combined with lab-made buffers provide higher yields in regard to RNA

about the efficacy of any method since FFPE tissue samples from the same block vary in tissue quantity. Thus, it may lead to false conclusions.

Decontaminated and regenerated silica-based spin columns prove to be equally effective in RNA purification as compared to new ones. However, we recommend that RNA samples obtained from reused columns should be used only for quantitative analyses and not for qualitative detection, as the risk of cross contamination still exists.

CONCLUSION

Silica-based spin-columns provide maximum yield and purity of the isolated RNA. Lab-made buffers provide efficacious purification of the RNA during washing cycles. Decontamination of spin columns with non-ionic detergent containing alkaline buffer and regeneration with acidic sodium acetate buffer allows them to be reused for subsequent extractions of RNA, as effectively as the new ones. In conclusion, we improved the efficiency of silica-based spin-columns in order to provide an effective and inexpensive way to perform RNA extractions from limited tissue samples, like FFPE.

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