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Original Article

An improved method for isolation of RNA from rat femur

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Abstract: Background: RNA isolation from ossified bone is a difficult and time-consuming process which often results in poor recovery of RNA. The yield is limited and might not be suitable for gene quantification studies by real time PCR. Methodology: The present study demonstrates RNA extraction from rat femur utilizing the silica column along with the trizol reagent. Quality of RNA was assessed by agarose gel analysis and its suitability for real-time PCR analysis was determined by β-actin Ct values. Results: The RNA isolated using silica columns in conjugation with trizol reagent resulted in higher yield of RNA and purity (A260/280=2.04; yield =1545.73 μg/ml) compared to the trizol method alone (A260/280=1.85; yield =571.2 μg/ml). Ct value of β actin obtained from RNA isolated by trizol method was higher than the Ct value obtained by trizol in conjugation with the column method (31.41 and 15.41 respectively). Conclusion: Combination of trizol along with silica column resulted in better quality and improved yield of RNA suitable for gene quantification by Real time PCR.

Keywords: Femur, rat, RNA, trizol

Introduction

Ribonucleic acid (RNA) based applications have revolutionized biomedical research to monitor gene modulation during the onset and progression of the disease [1]. Therefore, RNA extracted from the bone can be utilized for gene expression analysis, RNA-protein interactions, genetic network analysis and diagnostics and therapeutics of the disease [2, 3]. However, obtaining intact, high-quality RNA is challenging from bone as this tissue is highly mineralized and has very few cells entrenched in it.

Traditional methods for isolating RNA are based on the use of chaotropic agents such as guanidine thiocynate, guanidine hydrochloride and ammonium isothiocynate, which are able to destroy cell structure and inactivate cellular RNases. Commercially available agents are also used for RNA extraction, such as RNAzol (GeneCopoeia™) and trizol (Molecular Research Center), which contain high concentrations of guanidine thyocyanide (>4 M) and urea salts. There have been studies which have shown RNA extraction from mice femur bone [4] human articular cartilage [5] and human nucleus pulposus of intervertebral discs [6] using Tri reagent. However, trizol alone failed to yield good quality and quantity of RNA from mineralized tissues. Newer methods like silica-based matrices systems have also gained popularity in recent years for total RNA extraction. These commercial preparations, when used, facilitate RNA isolation from small quantities of samples and act by denaturing RNases, resulting in speedy recovery [7, 8]. A recent report by Frideriki Poutoglidou demonstrated RNA isolation method for rat bones utilizing RNAlater ice along with a simple laboratory homogenizer. Nevertheless, study lacks the proof of downstream application of the extracted RNA [4]. Another study done by Pedersen *et al*. has isolated RNA from mice mineralized bone utilizing Trizol reagent followed by purification by RNeasy columns [9] which resulted in good

quality and quantity of RNA. However, extracting RNA from mice bone is less tedious as it is less calcified and soft as compared to rat bone [10]. Studying rat bone remodeling is important because the ovariectomized model of the rat is approved by the FDA as a suitable model for postmenopausal osteoporosis research [11]. Therefore, we addressed this question and devised the protocol by combining trizol along with columns embedded with silica-based matrices for extracting RNA from rat bone. Our method facilitates the isolation of high quality and quantity of RNA from rat femur.

Materials and methods

Chemicals

All the chemicals used were of analytical or molecular biological grade and obtained from commercial sources. TRI reagent was purchased from Sigma (USA). Total RNA extraction kit was purchased from Invitrogen (USA). Deoxyribonuclease I (Amplification Grade) was purchased from Sigma (USA). RevertAidTM H Minus First Strand cDNA Synthesis kit was purchased from Fermentas (USA). FastStart SYBR Green Master was purchased from Roche. PCR primers were procured from Integrated DNA Technologies (USA). Routine chemicals like agarose, Tris, EDTA, bromophenol blue etc. were purchased from Himedia (India) and SRL (India).

RNA isolation

Six-month-old female Sprague Dawley rats (n=6) weighing between 125-150 g were procured from the Central Animal House of Postgraduate Institute of Medical Education and Research, Chandigarh, India. The experimental protocols were approved by the Institutional Ethics Committee (IAEC/87a) and conducted according to Indian National Science Academy (INSA) Guidelines for the use and care of experimental animals. The temperature of the animal room was maintained at 21±1°C, humidity 50-60% and a 12 h dark and light cycle. Rats were sacrificed, femur bones were harvested and, muscular tissue attached to the bones was removed using scalpel blade. The bones without flesh were snap frozen in liquid nitrogen and stored in a -80°C refrigerator prior to RNA isolation.

The bones were grounded into fine powder in a pre-cooled mortar and pestle filled with liquid nitrogen. The grounded powder was divided into two tubes and mixed with 1 ml TRIzol® Reagent per 50-60 mg tissue per tube. One tube was processed by TRIzol® Reagent (Sigma, USA) method and the other tube was processed by TRIzol® Reagent (Sigma, USA) in conjugation with the PureLink™ RNA Mini Kit (Invitrogen). Each tube was centrifuged at 12,000× g for 10 minutes at 2-8°C to remove debris. The supernatant was collected in a fresh tube and 0.1 ml of 1-bromo-3-chloropropane was added to it. Tubes were shaken vigorously for 30 seconds, and allowed to stand for 10 minutes at room temperature to ensure phase separation and complete dissociation of nucleoprotein complexes. The mixture was centrifuged at 12,000× g for 15 minutes at 2-8°C, which resulted in separation of the upper aqueous phase and the lower organic phase. After this, one tube was processed with the normal trizol method and the other tube was processed with the column method. For the trizol method, the upper aqueous phase was transferred to a fresh tube and 0.5 ml of 2-propanol was added to the tube. The sample was allowed to stand for 10 minutes at room temperature and then centrifuged at 12,000× g for 10 minutes at 2-8°C. The supernatant was discarded and the RNA pellet formed was washed twice with 1 ml of 75% ethanol. The pellet was air dried by keeping the lid of the tube open for a few minutes in a sterile environment. The pellet was dissolved in 50 µl RNase-Free water and stored at -80°C after spectrophotometric quantification.

For the column method, the upper aqueous phase containing the RNA was transferred to a fresh RNase-free tube and an equal volume of 70% ethanol was added. The sample was then processed through a Spin Cartridge from PureLink™ RNA Mini Kit, Invitrogen, containing a clear silica-based membrane to which the RNA binds. Impurities were effectively removed by subsequent washing of the column with wash buffers I and II provided in the kit, and centrifuging at 12000× g, 30 seconds at room temperature. Further, the columns were dried by centrifuging them at 12000× g, 1-2 minutes at room temperature and transferred to fresh tubes. 50 µl of RNase free water was added to the center of column and kept for 1-2 minutes.

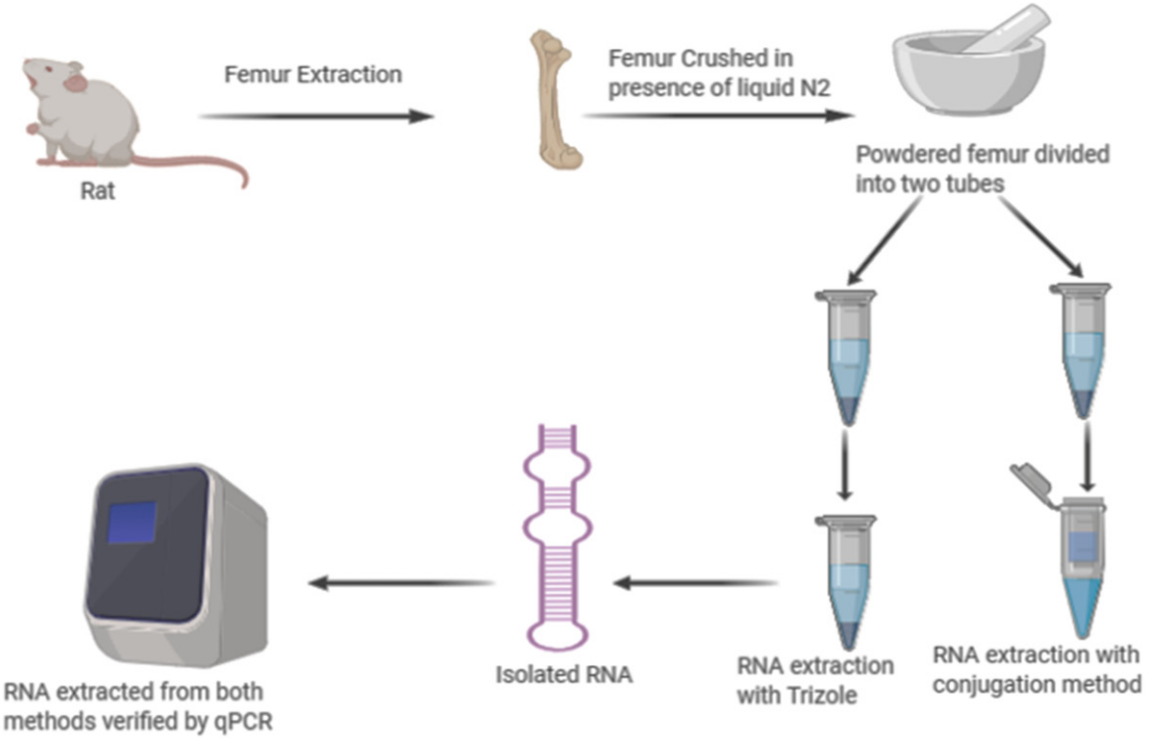

Figure 1. The graphical presentation of the protocol followed.

The columns were then centrifuged at 12000× g for 2 minutes at room temperature and purified RNA was eluted in 50 µl of RNase-Free water and stored at -80°C after spectrophotometric quantification. RNA samples obtained from both methods were treated with 1 unit of Deoxyribonuclease I (DNase I) (Sigma, USA) for 10 µg RNA for 15 minutes. One µl of stop solution was added and heated at 70°C for 10 minutes to inactivate DNase. The quality of RNA was checked on 1% TAE (Tris-acetate-EDTA buffer) agarose gel electrophoresis.

Messenger RNA was transcribed into cDNA using the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences, USA) following manufactures instructions. **Figure 1** shows the brief description of the protocol followed.

Quantitative real time PCR

The cDNA synthesized was used as a template to check gene expression of β-actin by SYBR green chemistry. Briefly, cDNA was mixed with SYBR green I master mix and primers at a final concentration of 0.5 µM in 10 µl of reaction volume. The forward and reverse primers used were 5'-CCCCATTGAACACGGCATT-3' and 5'-GATGGCTACGTACATGGCTGG-3' respectively. The reaction was performed on Lightcycler® 480 II instrument from Roche with an initial stage of 5 mins to activate DNA polymerase at 95°C followed by 40 cycles of denaturation at 95°C for 10 sec; annealing at 55°C for 20 sec; extension at 72°C for 20 sec. Melt curve analysis was performed from 55-95°C. A cycle threshold (Ct) value was generated for each sample. The specificity of the amplified product in each case was checked by melt curve analysis.

Statistics

Three independent experiments were performed to come to the conclusion. The significance of the experiments was calculated using a t-test in Microsoft Excel. The data are presented as a mean standard deviation. Significant was defined as a *P*-value of less than 0.05.

Results and discussion

RNA was isolated from the rat femur using trizol method and trizol in conjugation with a column

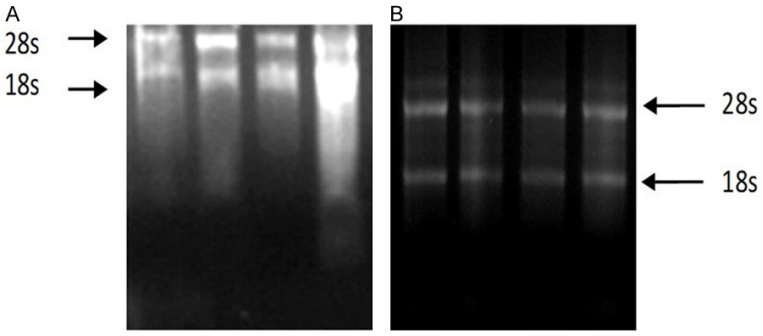

Figure 2. RNA quality in bone tissue. Total RNA was extracted from femur. (A) Total RNA was extracted from frozen femur using trizol method and (B) Total RNA extracted from frozen femur with trizol in conjugation with column method. RNA quality was visualized using agarose gel chromatography. Total RNA extracted showed 28 S and 18 S ribosomal RNA bands.

Table 1. Comparison of RNA yield and quality extracted from trizol alone and trizol in conjugation with column method. All the values were expressed in mean ± standard deviation (SD)

	Yield (µg/ml)#	A 260/280
Trizol method	571.2±40.68	1.85±0.09
Trizol in conjugation with column method	1545.73±47.42***	2.04±0.02

#50 mg of tissue was used to isolate total RNA, which was finally dissolved in 50 µl RNAse free water. ***P<0.0001; t-test was performed to calculate the significance difference between the yield of RNA extracted by trizol and trizol in conjugation with column methods.

method. Isolated RNA was treated with DNase I to remove any DNA contamination. **Figure 2** shows the quality of RNA isolated with both methods on agarose gel electrophoresis. RNA isolated by the trizol method was degraded and had salt contamination. However, the conjugation method yielded good quality RNA with no salt contamination (**Figure 2**). The yield obtained from the trizol method alone was significantly lower (P<0.0001) in the comparison to the yield obtained from conjugation method (571.2±40.68 and 1545.73±47.42 µg/ml respectively) (**Table 1**). The suitability of the bone RNA for downstream application was demonstrated by real-time PCR analysis, using β-actin specific primers. The Ct value of β-actin obtained from the trizol method was 31.41±1.36 whereas Ct value obtained from the conjugation method was 15.41±1.08 (**Figure 3**). The difference between the Ct values obtained by two different methods was highly significant (P<0.0001). Recently, Frideriki Poutoglidou *et al.* has shown RNA yield of 129.8 µg/ml from rat bone using RNAlater ice along with a simple homogenizer [4]. However, the quantity obtained is very low when compared to our protocol and the study lacks information regarding downstream application of the extracted RNA [4]. Another protocol developed by Rabecca Nance *et al.* has used canine phalanges bone cells to isolate RNA. The method developed to homogenize bone utilizes motor and pestle which was chilled by keeping it on dry ice. Later, bone powder was mixed with zirconium beads to homogenize bone [12]. Our method utilized a cryogenic grinding method using liquid nitrogen, which is important for RNA isolation from bone. It makes the bone brittle and facilitates grinding. It also inhibits RNase activity by maintaining a low temperature. The mineralized bones of mice have also been used for RNA isolation using Tri reagent with RNeasy columns, showing good quality of extracted RNA [9]. Nevertheless, mice bone is soft and less calcified when equated with rat bone and is not suitable for bone remodeling studies [10]. In addition, the FDA has approved the ovariectomized rat as an osteoporosis research model [11]. Our method of RNA extraction from rat bone demonstrates better yield and improved purity from small amounts of bone (50 mg) when compared with previous protocols (**Table 2**). The method developed in this study is simple, short, and does not require the use of any specialized instruments. In comparison to the trizol method alone, our method facilitates the recovery of higher quantity and quality of RNA by utilizing columns along with trizol. Therefore, this optimized protocol for RNA extraction from rat bone would help the research community to work progressively towards betterment of bone health.

Conclusion

The current study combines the capabilities of trizol to rupture cell structure, facilitating the release and binding of RNA to the column. RNA

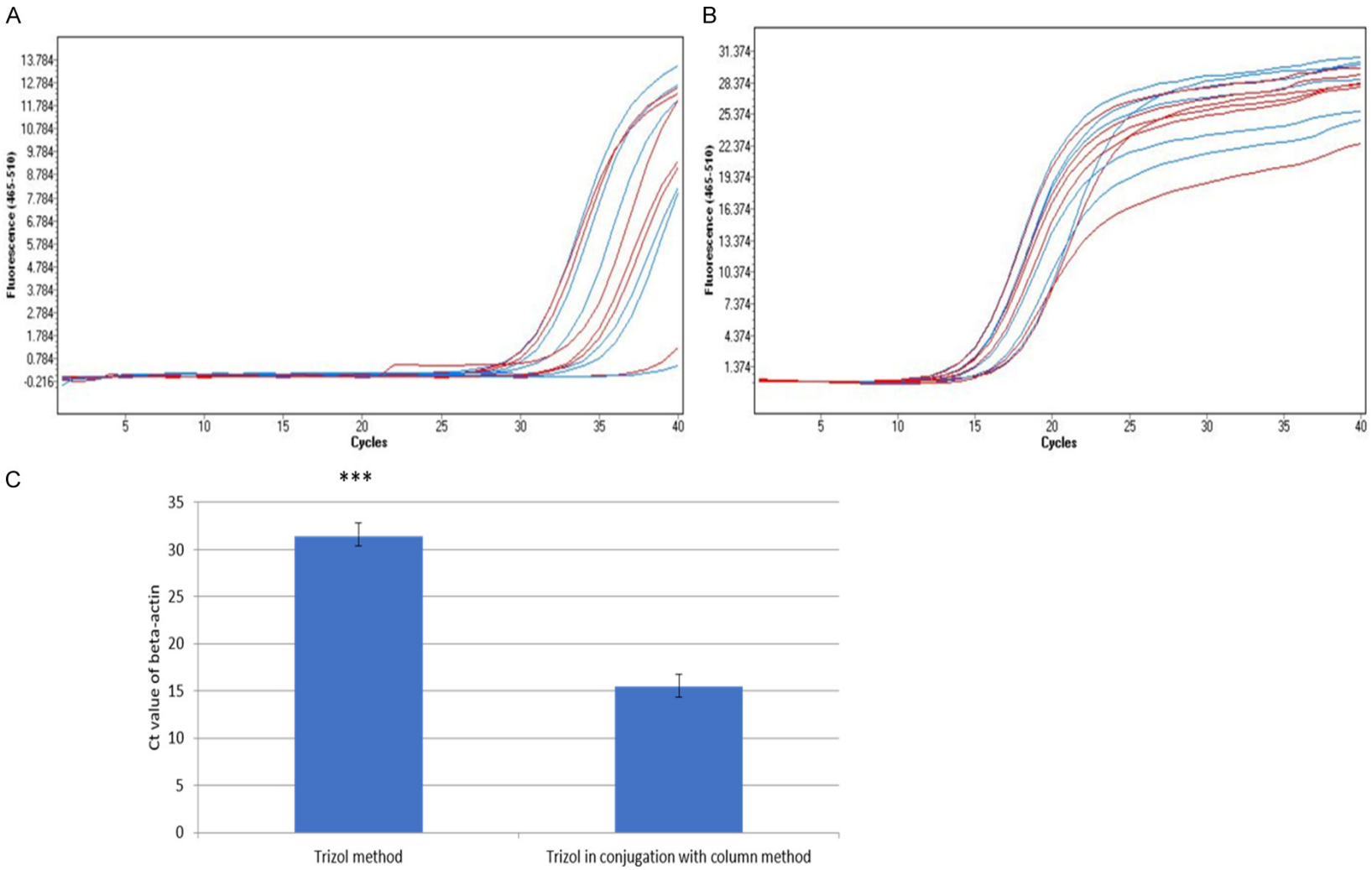

Figure 3. Amplification curve of β-actin made from RNA extracted from (A) trizol method and (B) trizol in conjugation of column method. (C) Ct value comparision between the two methods. ***P<0.0001; t-test was performed to calculate the significance difference between Ct values obtained for β-actin by two methods.

Table 2. Comparisons between various RNA isolation protocols from bone

Sample	Yield	Reference
Mice (solid joint)	26.0±7.76 µg	[13]
Canine (phalanges)	14.7 µg/g	[12]
Mice (femur)	Approx. 40 µg	[9]
Rat (femur and tibia)	102-109 µg/ml	[4]
Rat (femur)	1545 µg/ml	Present study

purified from our method can be used for digital PCR, Real-time PCR, northern blotting, array analysis, next-generation sequencing, and cDNA library construction. This improved isolation method could be a stepping stone towards unraveling the molecular mechanisms of various bone diseases.

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Disclosure of conflict of interest

None.

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