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RESEARCH ARTICLE

Optimized RNA isolation method for field-grown mature rice roots

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Abstract

High-quality RNA extraction was crucial for downstream functional genomics applications. Tissue-specific gene expression studies relied heavily on understanding molecular mechanisms at the cellular level. RNA isolation from mature field-grown rice roots posed significant challenges due to polysaccharides, secondary metabolites and other contaminants. The study contributed to development of a modified TRIzol-based RNA isolation protocol for mature field-grown rice roots. The TRIzol RNA extraction protocol was modified and compared its efficiency with four established methods: RNeasy Plant mini kit, CTAB-LiCl method, SDS method and TRIzol method. The key modifications included an increased extraction volume (1.5 mL per 100 mg tissue) and a higher ethanol concentration (80 %), along with streamlined steps to reduce processing time. The modified TRIzol method yielded an average of 537 ng/µL of high-quality RNA (A_{260}/A_{280} ~ 2) from mature field-grown rice root tissues. The RNA obtained using the modified TRIzol method was validated through cDNA synthesis and amplification of the housekeeping gene actin. Syber green-based RT-qPCR revealed a single peak in the melt curve analysis, confirming the specificity of primers for cDNA binding. The optimized TRIzol protocol offered a faster, cost-effective and user-friendly approach for isolating high-quality RNA from mature field-grown rice roots compared to existing methods. Due to its high efficiency and reliability, this protocol serves as a robust and practical tool for functional genomics and molecular biology research involving challenging plant tissues.

Keywords: CTAB; gene expression; rice; RNA isolation; SDS; TRIzol method

Introduction

Rice (*Oryza sativa* L.) is a globally significant staple crop and a key model plant for understanding various biological processes. In the context of ongoing climate change, crop improvement programs in rice required a comprehensive understanding of genes influencing yield, nitrogen use efficiency and stress tolerance under field conditions. However, gene expression in rice roots, particularly under field conditions, had been less explored compared to the aerial parts of the plant.

Roots, the below-ground organ, played a pivotal role in water and nutrient intake, plant metabolism and anchoring, making them the focal point of interest for plant biologists (1, 2). Investigating root function at the molecular level under real field conditions had the potential to drive advancements in research on root structure and functionality, thereby aiding crop improvement initiatives.

High-quality, DNA-free RNA was essential for a wide range of plant molecular biology applications, including reverse transcription polymerase chain reaction (RT-PCR), quantitative real-time PCR (qPCR), cDNA library construction, northern blotting, RNase protection assays, *in situ* hybridization and comprehensive gene expression profiling. However, isolating high-quality RNA from plants was often challenging due to the biochemical complexity of plant tissues, especially those rich in polysaccharides, proteins and secondary metabolites (3). Polysaccharides, in particular, tended to co-precipitate with RNA in low ionic strength buffers, acting as enzyme inhibitors and compromising RNA yield and purity. Phenolic compounds could oxidize and covalently bind to nucleic acids, further reducing RNA integrity (4).

Moreover, the process of physically separating roots from compacted or moist soils often lead to mechanical damage and tissue degradation, especially if immediate freezing or preservation was not feasible. Rapid and efficient sample handling was, therefore, critical to minimize RNA degradation by endogenous RNases, which were highly active in root tissues.

Extracting RNA from below-ground tissues such as roots and rhizomes of field-grown plants proved particularly difficult, especially in mature roots (5). Despite the availability of numerous RNA extraction protocols for various plant species and tissue types, transcriptomic studies focusing on root tissues from field-grown plants remained limited (6–9). Since transcriptomic workflows commenced with total RNA extraction, the acquisition of high-quality RNA was considered a critical initial step. The reliability of gene expression data was directly linked to the integrity and purity of the starting RNA (10). However, attaining the required RNA remained challenging even with the modern isolation techniques.

Commercial RNA extraction kits facilitated plant molecular biology research. However, extracting high-quality RNA from polysaccharide-rich tissues remained a persistent challenge. Performance of kits varied depending on plant species, genotype, tissue type and biochemical composition (11, 12). The RNA yield from rice roots grown under paddy conditions declined progressively with plant maturation. After the panicle formation stage, commercial kits failed to extract sufficient RNA from root tissues, particularly during rice's tillering to ripening stages (13).

This study aimed to standardize a simple, rapid and cost-effective RNA extraction protocol for mature rice roots grown under field conditions. The conventional TRIzol method was modified and its efficiency was compared with other widely used methods, including the RNeasy Plant Mini Kit (Qiagen), the CTAB-LiCl method, the SDS-LiCl method and the standard TRIzol protocol (12, 14, 15).

Materials and methods

Collection of plant materials

Mature root samples (after the panicle initiation stage) were collected from the rice variety *Jyothi* from the rice field of the College of Agriculture, Vellayani (Fig. 1). Roots were carefully excavated using a shovel and soil was removed by thoroughly washing them with tap water. After the initial washing, the roots underwent 2 additional rinses. Subsequently, the roots were gently dried, wrapped in aluminium foil frozen using liquid nitrogen and stored at -80 °C until use.

Preparations for RNA isolation

Solutions were prepared using diethyl pyrocarbanate (DEPC) treated water (DTW, 0.1 %) to ensure RNase free conditions. To inactivate RNase, pipette tips, centrifuge tubes and the mortar and pestle were immersed in DEPC-treated water overnight. After being drained, they were sterilized by autoclaving at 121 °C for 40 min. Before use, the autoclaved mortar and pestle were pre-chilled in a refrigerator and cleaned with RNase OUT (G-biosciences) to eliminate residual RNase. Plasticware, glassware and gel tank were treated with 3 % H_2O_2, followed by a rinse with sterile DTW prior to use. Molecular biology grade reagents were used throughout the preparation and utmost care was taken by consistently wearing and changing gloves. RNase OUT (G-biosciences) was applied to ensure the absence of RNase in the working area. The RNA isolation protocols used in the study were detailed below. All RNA isolations and subsequent cDNA confirmations were performed in 2 independent replicates to ensure reproducibility.

Fig. 1. Morphology of roots from field-grown rice after the panicle initiation stage.

RNeasy Plant mini kit (Qiagen)

RNA was extracted according to the manufacturer's guidelines. Finely powdered mature roots (100 mg) were extracted with 450 µL of provided extraction buffer. Following the manufacturer's protocol, RNA was eluted in 30 µL of RNase-free water and stored at -80 °C.

CTAB-LiCl method

Roots (400 mg) were finely ground into a powder under liquid nitrogen and transferred to RNase-free 2 mL microcentrifuge tubes. To this 1 mL prewarmed CTAB extraction buffer (2 % CTAB, (w/v), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 2 % PVP) and 20 µL β-Mercaptoethanol (ME) were added. The sample was thoroughly vortexed and then incubated at 65°C for 20 min. Following the incubation, each sample was mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 15000 x g for 10 min at 4 °C. The aqueous phase was carefully transferred to a fresh 2 mL RNase-free microcentrifuge tube and the extraction was repeated once using an equal volume of chloroform : isoamyl alcohol. The resulting supernatant was combined with one-third volume of 8 M lithium chloride (LiCl) in a 1.5 mL RNase-free microcentrifuge tube and incubated overnight at -20 °C for RNA precipitation. The RNA pellet, obtained by centrifugation at 15000 x g for 10 min at 4 °C, was washed with 1 mL of pre-chilled 70 % ethanol, resuspended in 30 µL of RNase-free water and stored at -80 °C (12).

SDS RNA extraction method

Mature roots (400 mg) were finely ground into a powder using a chilled mortar and pestle with liquid nitrogen. Subsequently, 3 mL of extraction buffer containing 150 mM Tris base (hydroxymethyl-hydrochloride), 4 % (w/v) SDS, 100 mM EDTA (pH 7.5, adjusted with saturated boric acid), 60 µL β

-ME (2 %, v/v) and 3 % (w/v) PVP-40 was added to ground tissue. Approximately 750 µL of the homogenate was transferred to an RNase-free 2 mL microcentrifuge tube and precipitated using 66 µL of 5 mM potassium acetate and 150 µL of absolute ethanol. An equal volume of chloroform-isoamyl alcohol (49:1, v/v) was then added, the mixture was vortexed and centrifuged at 16000 x g for 20 min at room temperature. The resulting supernatant was transferred to fresh 2 mL RNase-free tubes and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added.

After vortexing for 10 sec, the sample was centrifuged at 16,000 x g for 15 min at room temperature. The aqueous phase was transferred to 1.5 mL RNase-free microcentrifuge tube and 3 M LiCl was added, mixed gently by inversion and incubated overnight at 4 °C for RNA precipitation. Following incubation, the RNA pellet was obtained after centrifugation at 16,000 x g for 20 min at 4 °C washed with 1mL pre-chilled 70 % ethanol, air -dried at room temperature under sterile laminar airflow. The final pellet was resuspended in 30 µL of RNase-free water and stored at -80 °C (15).

TRIzol method

Root samples (400 mg) were finely ground using liquid nitrogen, followed by the addition of 4 mL of RDP Trio™ reagent (Himedia) and homogenization thoroughly. The homogenate was incubated at room temperature for 5 min and then transferred to a pre-chilled 2 mL microcentrifuge tube. Subsequently 0.2 mL of chloroform was added and the mixture was vigorously shaken for 15 sec, incubated on ice for 15 min and then centrifuged at 10000 x g for 20 min at 4 °C. The RNA-containing aqueous phase was carefully transferred to fresh 1.5 mL RNase-free microcentrifuge tubes, mixed with 1 mL of pre-chilled isopropanol and stored at -20 °C for at least 1 hr to facilitate RNA precipitation. The resulting pellet, obtained after centrifugation at 10000 x g for 20 min, was washed with 1 mL of pre-chilled 75 % ethanol. Following an additional centrifugation at 7500 x g for 5 min at 4 °C, the supernatant was discarded and the pellet was air-dried in a laminar flow hood. The dried pellet was then dissolved in 30 µL of RNase-free water. Finally, the RNA was incubated at 65 °C for 10 min and stored in a -80 °C for downstream applications(14).

Modified TRIzol method

Mature roots (400 mg) were ground to a fine powder with liquid nitrogen and transferred to a 15 mL RNase-free centrifuge tube along with liquid nitrogen. RDP Trio™ reagent (Himedia), was added to the chilled ground root powder at ratio of 1.5 mL per 100 mg of tissue, totaling 6 mL. The sample was gently pipetted up and down to ensure proper mixing and was then incubated at room temperature for 5 min. From this mixture, 1 mL aliquots were transferred to pre-chilled 2 mL RNase-free centrifuge tubes. Chloroform (200 µL per 1 mL of the sample) was added to each tube, which were then shaken vigorously for 30 sec and incubated on ice for 5 min. Subsequently the tubes were centrifuged at 14000 x g for 10 min at 4 °C. The resulting supernatant was transferred to fresh 1.5 mL RNase-free microcentrifuge tube. Pre-chilled isopropanol (70 % of the supernatant obtained) was added and the mixture was incubated on ice for 5 min. The samples were then centrifugated at 12000 x g for 10 min at 4 °C. The resulting pellet obtained was washed with 1mL of pre-chilled 80 % ethanol at 7500 x g for 7 min at 4 °C. The pellet was air-dried in a laminar flow hood for 10 min to eliminate the residual ethanol and was then resuspended in 30 µL RNase-free water. The extracted RNA was stored at -80 °C freezer for further use.

RNA quality and quantity analysis

The purity and integrity of the RNA were assessed using a NanoDrop™ Lite Spectrophotometer (Thermo Scientific) and were visualized on 2 % (w/v) agarose gels in 1 X TBE buffer. An 8 µL sample with 2 µL RNase-free loading dye (5 X, Himedia) was run on the gel for every RNA sample.

cDNA preparation

One microgram of RNA, extracted using the modified TRIzol method, was used for cDNA synthesis using the Verso cDNA kit (Thermo Scientific) following the manufacture's instruction. Successful cDNA synthesis was confirmed by PCR amplification of the rice actin gene using gene-specific primers (Forward: 5'-CAGCCACACTGTCCCCATCTA-3'; Reverse: 5'-AGCAAGGTCGAGACGAAGGA-3') (16). The PCR product was resolved on a 1.8 % (w/v) agarose gel in 1 X TBE buffer. For each sample, 8 µL PCR product was mixed with 2 µL 5 X loading dye (Himedia) and electrophoresed and the results were documented using a Bio-Rad Gel Doc Unit (using Quantity One software).

Real-Time PCR analysis

To validate the product specificity, melt curve analysis was done using syber green based RT-qPCR (CFX96 Real time system, Bio-Rad) by amplifying the housekeeping gene (*actin*) of rice. The cDNA synthesized from RNA extracted using our optimized TRIzol method was utilized for RT-qPCR.

Results and Discussions

Fig. 2 and Table 1 present the yield and purity of RNA isolated from mature rice roots grown under field conditions. Among the 5 methods evaluated, the modified TRIzol method produced significantly higher quantities of high-quality RNA (537 ng/µL) compared to the original TRIzol method (121.2 ng/µL), RNeasy Plant Mini Kit (31.1 ng/µL), CTAB-LiCl method (9.3 ng/µL) and SDS RNA extraction method (23 ng/µL). Distinct 28S, 18S and 5.8S rRNA bands were observed on 2 % (w/v) agarose gels with no signs of DNA contamination or RNA degradation (Fig. 2). The RNA extracted using this method also showed an A_{260}/A_{280} ratio of approximately 2.0, indicating minimal contamination from proteins, polysaccharides, or polyphenols (Table 1). In contrast, the other methods produced lower-quality RNA.

RNA yield and quality varied among plant species depending on tissue type and developmental stage (17). Extracting RNA from field-grown rice roots was particularly challenging due to the low RNA concentration in mature root tissues. This was attributed to the lysigenous aerenchyma, which resulted from programmed cell death in a significant portion of the roots (18). Furthermore, the presence of secondary metabolites, polyphenols and polysaccharides complicated the process. Phenolic compounds such as lignin and humic acid, which accumulated during the degradation of organic matter in field conditions, further hindered RNA extraction (13).

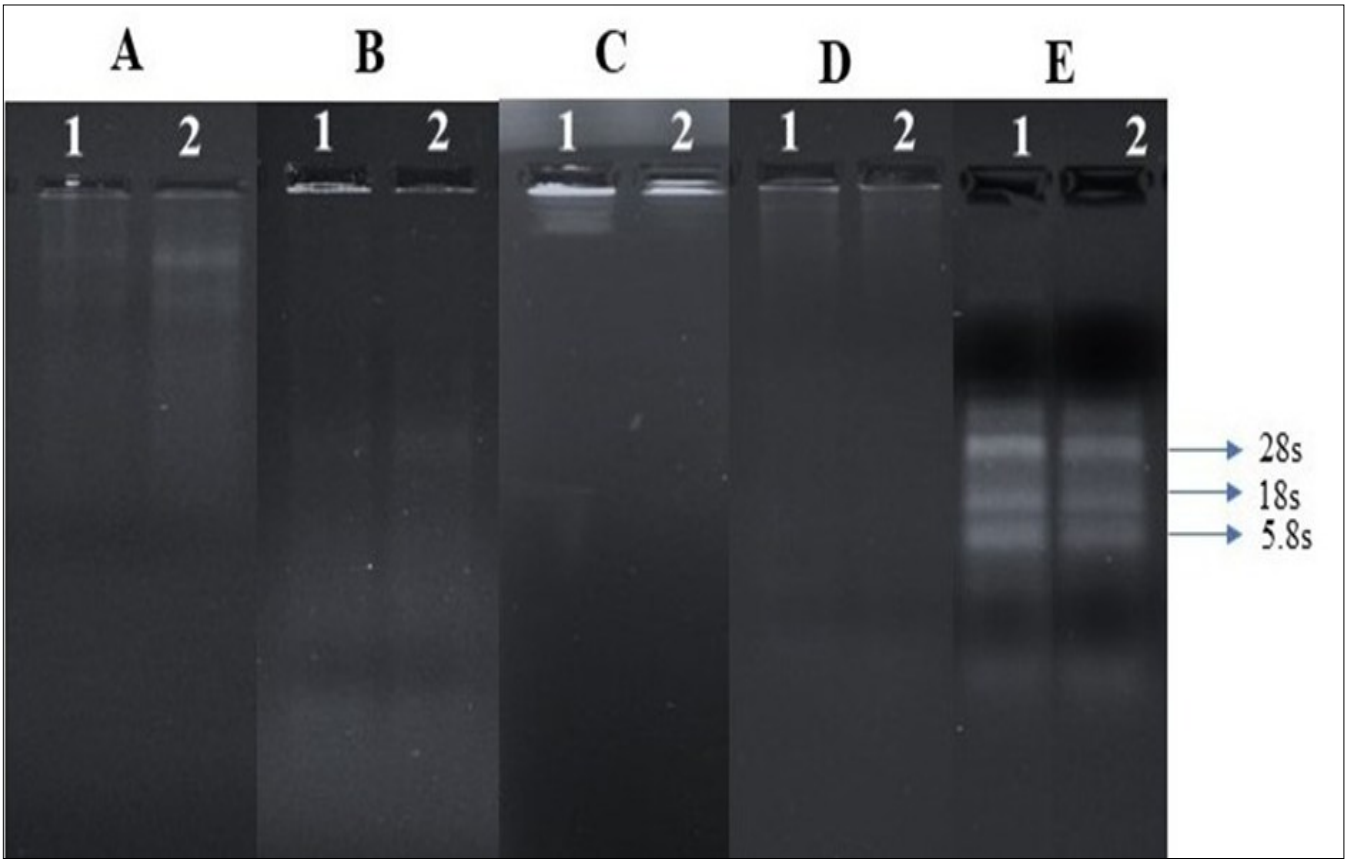

Fig. 2. Total RNA extracted from field-grown mature rice roots. (A) TRIzol method, (B) RNeasy Plant mini kit, (C) CTAB-LiCl method, (D) SDS method, (E) Modified TRIzol method. Lanes 1 and 2 represent biological replicates of RNA samples extracted using each method.

Table 1. Quality and purity of total RNA extracted using different methods from field-grown mature rice roots.

Protocols	Purity		RNA concentration (ng/µL)
	A_{260}/A_{280}	A_{260}/A_{230}	
TRIzol method	1.83	1.6	121.2
RNeasy Plant mini kit (Qiagen)	1.75	1.45	31.1
CTAB-LiCl method	1.36	0.9	9.3
SDS method	1.50	0.6	23.0
Modified TRIzol method	2.03	2.1	537.0

The values are average of 2 replications.

TRIzol was a widely used reagent for RNA extraction from diverse plant materials due to its simplicity, short processing time and ease of use (19). However, the results varied according to the tissue type. Although commercial RNA isolation kits offered simplicity and speed, they often failed to yield sufficient RNA from field-grown rice roots and were not cost-effective for large-scale applications. Studies highlighted their limited success in isolating RNA from root tissues (13, 20). Therefore, this research focused on devising a swift and economical method for RNA extraction, specifically suited for mature rice roots cultivated in field conditions.

Time was a critical factor influencing RNA yield, particularly for root tissues collected under field conditions. The process of uprooting, washing and isolating RNA from field -grown rice roots was labour-intensive and time-consuming. Washing fibrous rice roots thoroughly after field collection was especially challenging and was found to influence RNA yield and gene expression. For instance, prolonged washing altered the expression of aquaporin genes within just 5 min (21). Even with skilled assistance, cleaning rice roots after the panicle initiation stage required 1.5 to 2.5 min. While the washing step could not be cannot be significantly shortened, optimizing the RNA isolation process offered an opportunity to reduce overall processing time.

To address these challenges, the TRIzol protocol was modified by streamlining steps such as centrifugation and incubation (14). Additionally, the ethanol concentration was increased to 80 % to expedite RNA pellet drying. These modifications allowed RNA isolation to be completed within 90 min for 3 samples, although the duration depended on the researcher's experience. In comparison, the CTAB-LiCl and SDS methods tested were labour-intensive and time-consuming, despite their established efficacy in isolating RNA from roots of other crops (22–24). However, these methods yielded suboptimal results for rice roots.

To validate the purity and integrity of RNA isolated using the refined TRIzol method, cDNA was synthesized and subjected to PCR amplification of the housekeeping gene *actin*. The expected 67 bp band was observed on a 2 % (w/v) agarose gel (Fig. 3). RT-qPCR using cDNA derived from this RNA yielded a single peak in the melt curve analysis, confirming primer specificity for cDNA binding and the absence of PCR inhibitors (Fig. 4). These results demonstrated the efficiency of the modified TRIzol method in consistently yielding RNA of sufficient quality and integrity for downstream gene expression analysis.

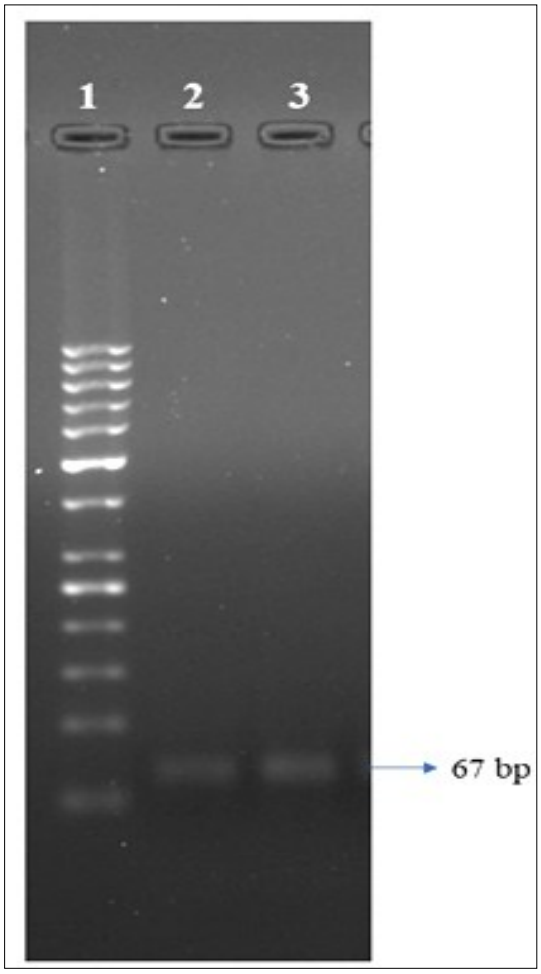

Fig. 3. Lane 1: 50 bp ladder; Lanes 2 and 3: *actin* gene amplicon of 67 bp.

Conclusion

The modified TRIzol method proved to be highly effective for extracting good quantities of high-quality RNA from mature rice roots grown under field conditions. By systematically optimizing the protocol, this method minimised both isolation time and cost while outperforming commercial RNA isolation kits and other tested methods. The demonstrated efficacy of the modified TRIzol protocol highlights its potential as a reliable tool for investigating root specific gene expression, particularly for studies focused on nutrient utilization efficiency and abiotic stress responses.

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Authors' contributions

EJ conducted the experiment, collected and analyzed data and prepared the manuscript. SKB conceived the idea, supervised the work and interpreted the data. SA, SPP, BR and RS assisted in planning and designing the experiments, supervision and interpretation of data. MDD assisted in data analysis and editing of the manuscript.

Compliance with ethical standards

Conflict of interest: All authors declare that they have no conflict of interest.

Ethical issues: None

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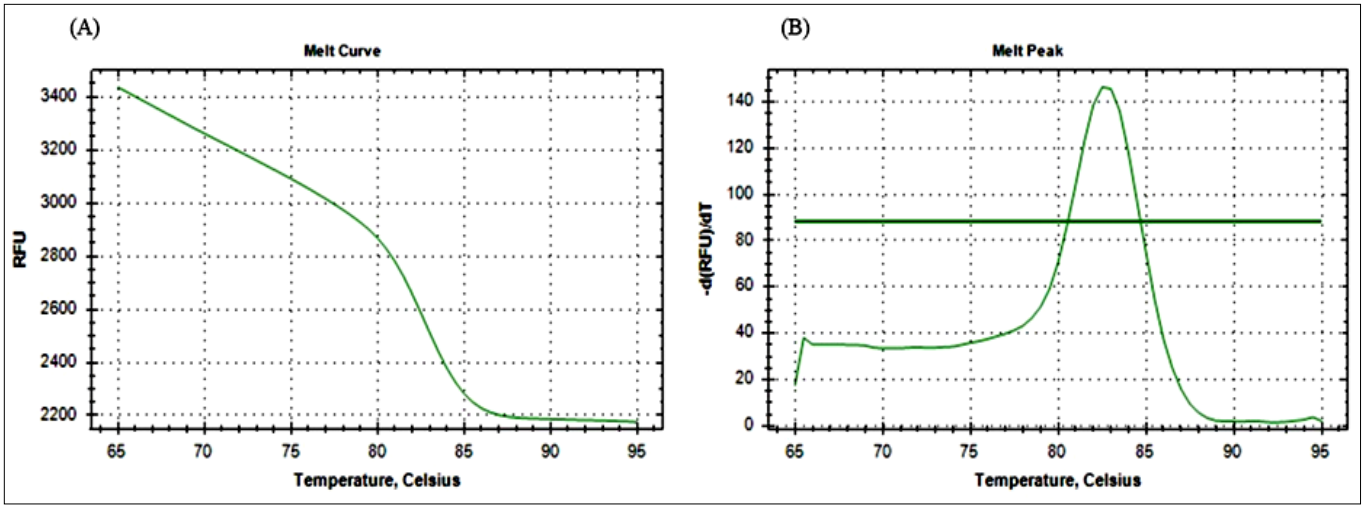

Fig. 4. RT-qPCR assay using the cDNA from RNA extracted with the modified TRIzol method. (A) Melt curve and (B) Melt peak of *actin* gene showing a single specific amplification product.

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