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Short Communication

# Rapid two-stage method for extracting high-quality RNA from sunflower seeds (*Helianthus annuus* L.)

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## Abstract

**Importance of the work:** An efficient extraction method is crucial to obtain high-quality RNA which is an essential prerequisite for next-generation sequencing (NGS) and other downstream applications.

**Objectives:** To provide a suitable RNA extraction protocol for sunflower seeds that contain high levels of polysaccharides, secondary metabolites and lipids.

**Materials & Methods:** Total RNA was extracted from seed samples following classical and modified protocols, as well as using commercially available reagents. The two-stage extraction method involved the use of an optimized protocol for RNA extraction from oilseeds combined with the use of the TRIzol™ reagent as the second extraction buffer.

**Results:** The extraction protocols tested either failed or yielded low-quality RNA samples. The optimized two-stage RNA extraction method presented was completed within 4 h with yields as high as 629–740 ng/μL. The observed absorbance ratios ($A_{260}/A_{280} \geq 2.00$; $A_{260}/A_{230} \geq 2.00$) and RNA integrity number (6.4–8.2) indicated the high-quality of the isolated RNA.

**Main finding:** The optimized two-stage method was able to extract high-quality total RNA that can be used as a robust template for real-time reverse transcription-quantitative polymerase chain reaction analysis and NGS library construction.

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## Introduction

Advances in molecular biology have led to numerous studies in transcriptomics that have revealed specific insights on species metabolism, diversity and evolution (Zhang et al., 2021). Often described as a gold standard, the use of real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is primarily the method of choice for RNA quantification (Nolan et al., 2006). The rapid development of next-generation sequencing (NGS) technology has also made RNA sequencing (RNA-seq) a common approach for transcriptome analysis (Chowdhury et al., 2020). However, transcriptomic technologies strictly require high-quality RNA to facilitate downstream applications, with sample preparation and RNA extraction being critical steps in obtaining substantial and accurate data on differential gene expression and quantification using RNA-seq (Wolf, 2013).

Sunflower seeds are economically valued for their high oil content (Rocha et al., 2014). Sunflower plants are produced from seeds; therefore, germinating high-quality seeds is very important. However, its susceptibility to stress often results in poor seed germination and low yield. Understanding the underlying molecular mechanisms influencing seed quality and vigor would be advantageous in improving yield and enhancing stress tolerance. Thus, PCR-based methods and RNA-seq analysis have been performed on sunflower seeds. The reported high-quality sunflower genome and transcriptome (Badouin et al., 2017) promoted the start of several RNA-seq experiments using various sunflower cultivars. The increasing demand for high-quality RNA from sunflower seeds is challenged by the relatively low RNA yield, which has been attributed to the high levels of polyphenols, polysaccharides, protein, lipids and secondary metabolites in sunflower (Xu et al., 2010; Rayani and Nayeri, 2015). Phenolic compounds can bind to proteins and nucleic acids to form complexes, whereas polysaccharides remain in the final extract as contaminants; both may interfere with subsequent applications, such as PCR or NGS (Salzman et al., 1999; Footitt et al., 2018). Sunflower seeds are composed of up to 50% oil depending on the genotype (Salunkhe et al., 1992), thereby making RNA extraction highly variable.

Commercially available RNA extraction kits and reagents are dependent on the nature of the samples. The extraction of high-quality RNA in large amounts is not always guaranteed by these kits, especially when seeds have been subjected to postharvest treatments like seed priming. Several published protocols for RNA extraction have addressed the high levels of potential contaminants in seeds (Li and Trick, 2005; Birtic and Kranner, 2006; Ma and Yang, 2011; Mornkham et al., 2013; Rayani and Nayeri, 2015; Footitt et al., 2018). However, these methods are complex, time-consuming and occasionally inapplicable to sunflower seeds. The current study investigated an optimized two-stage method adapted from the method of Li and Trick (2005) that was combined with the use of the TRIzol™ reagent for the rapid and robust extraction of high-quality RNA from sunflower seeds for downstream applications.

## Materials and Methods

### *Seed materials*

$F_1$ hybrid sunflower seeds (*Helianthus annuus* L. cv. Aguara 6) were obtained from a local seed company in Thailand and stored in the cold room (0±2 °C, 55±5% relative humidity). The pericarp (hull) of the seeds was only removed immediately before RNA extraction.

### *Total RNA extraction*

Total RNA extraction was performed independently for each sunflower seed sample (A, B, C, D, E, F) with four technical replicates per sample. All seed samples were randomly selected from the same seed lot. Prior to extraction, all non-plastic materials were surface-washed with 0.1% diethylpyrocarbonate (DEPC)-treated water and sterilized by autoclaving. Likewise, stock solutions and reagents were prepared with DEPC-treated water and sterilized. The subsequent steps of RNA extraction were performed in 1.5 mL RNase-free microcentrifuge tubes under a fume hood.

Peeled sunflower seeds were ground into fine powder using liquid nitrogen in a mortar and pestle. The frozen powder was immediately transferred to a microcentrifuge tube and added with the respective extraction buffers described in the literature.

For the modified cetyltrimethyl ammonium bromide (CTAB) method (Chang et al.,1993), 100 mg of the frozen powder were mixed with 600 µL warm extraction buffer (2% CTAB; 2% PVP30; 0.1 M Tris-HCl, pH 8.0; 0.025 M ethylene-diamine-tetraacetic acid (EDTA); 2% β-mercaptoethanol, volume per volume (v/v), mixed just before use). The mixture was extracted twice with an equal volume of chloroform and isoamyl alcohol (24:1) and centrifuged at

15,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new tube, mixed with 8 M LiCl and incubated overnight at 4 °C. The next day, the pellet was obtained via centrifugation at 12,000 rpm for 30 min at 4 °C and then dissolved in 500 µL SSTE buffer (1 M NaCl; 0.5% SDS; 0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA, pH 8.0). The solution was mixed once with an equal volume of chloroform- isoamyl alcohol and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant was mixed with ethanol and incubated at -20 °C for 30 min. The pellet was obtained via centrifugation at 15,000 rpm for 30 min at 4 °C, washed with 80% ethanol and allowed to dry before re-suspending in RNAse-free water.

For the modified Ma and Yang method (Mornkham et al., 2013), 30 mg of the frozen powder were added with 900 µL of extraction buffer (8 M LiCl; 2% PVP40, w/v; 5% β-mercaptoethanol, v/v, mixed just before use) and 350 µL ethanol, vortexed and incubated at room temperature for 5 min. The impurities were removed through the addition of 100 µL chloroform, gentle mixing and centrifugation at 5,000 rpm for 3 min at 4 °C. The pellet obtained was dissolved in 550 µL solubilization buffer (1.4% SDS, w/v; 0.075 M NaCl; 0.025 M EDTA; 2% β-mercaptoethanol, v/v, mixed just before use). The chloroform extraction step was repeated. The supernatant obtained was transferred to a new tube and mixed with 500 µL TRIzol™ reagent and 100 µL chloroform. After mixing thoroughly, the sample was incubated at room temperature for 2 min, and centrifuged at 12,000 rpm for 10 min at 4 °C. The aqueous phase was transferred to a new tube, mixed with an equal volume of isopropanol and incubated at -20 °C for 15 min. The pellet was obtained via centrifugation at 12,000 rpm for 10 min at 4°C, air-dried and re-suspended in RNAse-free water.

For the modified Li and Trick method A (Osio et al., 2019), 100 mg of the frozen powder were added with 800 µL of extraction buffer (0.1 M Tris-HCl, pH 8.0; 0.15 M LiCl; 0.05 M EDTA, pH 8.0; 1.5% SDS, w/v; 0.015% β-mercaptoethanol, v/v, mixed just before use) and thoroughly mixed. Then, the sample was added with 500 µL phenol-chloroform (1:1), mixed via inversion and centrifuged at 13,000 rpm for 15 min at 4 °C. The aqueous phase was incubated with an equal volume of isopropanol at -20 °C for 1 h and were centrifuged at 13,000 rpm for 15 min at 4 °C. The resulting pellet was washed with 800 µL 70% ethanol, air-dried and re-suspended in RNAse-free water.

For the modified Li and Trick method B (Mornkham et al., 2013), 30 mg of the frozen powder was added with 750 µL of extraction buffer (0.1 M Tris-HCl, pH 8.0; 0.15 M LiCl; 0.05 M EDTA; 1.5% SDS, w/v; 11.25 µL β-mercaptoethanol, mixed just before use) and thoroughly mixed. The impurities were removed by adding 750 µL phenol-chloroform (1:1), mixing gently and centrifuging at 12,000 rpm for 10 min at 4 °C. The supernatant was collected and the phenol-chloroform extraction step was repeated. The supernatant obtained was mixed with 500 µL TRIzol™ reagent and incubated at room temperature for 10 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the sample, mixed gently and centrifuged at 12,000 rpm for 10 min at 4 °C. The aqueous phase was transferred to a new tube, mixed with an equal volume of isopropanol and 200 µL 1.2 M NaCl and incubated at -20°C for 15 min. The pellet was obtained via centrifugation at 10,000 rpm for 10 min at 4 °C, washing carefully with 70% ethanol and re-suspending in RNAse-free water.

For the Li and Trick method (2005) combined with TRIzol™ reagent, 50–100 mg of the frozen powder were mixed with 400 µL of extraction buffer I (0.1 M Tris-HCl, pH 8.0; 0.15 M LiCl; 0.05 M EDTA; 1.5% SDS, w/v; 1.5% β-mercaptoethanol, mixed just before use). After vigorous mixing, 250 µL phenol-chloroform (1:1) were added and the sample was centrifuged at 13,000×*g* for 15 min at 4 °C. The aqueous phase (approximately 250 µL) was transferred to a new tube and mixed with the TRIzol™ reagent as the second extraction buffer, following the manufacturer's instruction. The recovered supernatant (approximately 450 µL) was mixed with 300 µL isopropanol and 250 µL 1.2 M NaCl, and incubated on ice for 15 min. The samples were centrifuged at 13,000×*g* for 15 min at 4 °C and the obtained pellet was washed with 400 µL 70% ethanol, dried for 15–20 min at room temperature and re-suspended in RNAse-free water.

For the protocol with Plant RNA reagent (Invitrogen; Thermo Fisher Scientific; Waltham, MA, USA), RNA extraction was performed following the manufacturer's instructions.

For the optimized two-stage method presented in the current study, the frozen powder (100 mg) was added with 800 µL extraction buffer (0.1 M Tris-HCl, pH 8.0; 0.15 M LiCl; 0.05 M EDTA, pH 8.0; 1.5% SDS, w/v; 1.5% β-mercaptoethanol, v/v, added immediately prior to use) at room temperature (25±2 °C), and mixed vigorously using a vortex for 1 min. The mixture was added with 500 µL phenol-chloroform (1:1), mixed thoroughly by inverting the tubes several times and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new tube, added with an equal volume of the TRIzol™ reagent, vortexed briefly for 10 s and incubated at room temperature for 10 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added before the solution was

mixed by inversion and centrifuged at 12,000 rpm for 1 min at 4 °C. The resulting aqueous phase (approximately 500 µL) was collected into a new tube and added with an equal amount of isopropanol and 200 µL 1.2 M NaCl. The solution was incubated at -20 °C for 15 min and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was carefully discarded, and the remaining pellet was washed with 800 µL 70% ethanol. The pellet was air-dried, dissolved in 40 µL RNAse-free water (Qiagen; Hilden, Germany) and stored at -80°C until use.

For the DNase treatment, each RNA sample was mixed with 178 µL DEPC-treated water, 20 µL DNase buffer and 2 µL RNase-free DNase I (Qiagen) followed by incubation for 1 h at 37°C. When necessary, the chloroform-isoamyl alcohol step or the DNAse treatment was repeated to remove any remaining contaminants.

### *RNA yield, purity and integrity assessment*

The RNA concentration and quality of the samples (A, B and C) were determined by measuring the absorbance at 230 nm, 260 nm and 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific; Wilmington, DE, USA). RNA quality was evaluated based on the absorbance ratios ($A_{260}/A_{280}$ and $A_{260}/A_{230}$) while RNA integrity was visualized using 1% agarose gel electrophoresis. The gels were stained with MaestroSafe™ Nucleic Acid loading dye (MaestroGen; Hsinchu, Taiwan) and viewed under ultraviolet light using the Gene Genius Bioimaging System (Syngene; Frederick, MD, USA). To verify the spectrophotometric data, the quality and integrity of the RNA samples (D, E, F) were determined using an RNA Pico 6000 Chip Kit and a 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA).

### *cDNA synthesis, real-time reverse transcription-quantitative polymerase chain reaction analysis and library preparation*

To test the usability of the RNA extracts for downstream applications, the obtained total RNA from each sample (A, B, C) was reverse-transcribed using the Invitrogen SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific; Carlsbad, CA, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized with oligo$(dT)_{20}$ and amplified using primers for the *actin* gene (Ramu et al., 2016). The expected product size was 224 bp and the sequences of the forward and reverse primers were 5′-AGCTGCTGGTATTCACGAGACC-3′ and 5′-TCGATCCTCCGATCCAGACACTG-3′, respectively. The RT-qPCR experiment was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories; Hercules, CA, USA) in a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories; Hercules, CA, USA), with the following amplification conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplicon dissociation curves were recorded after 40 cycles by heating from 60 °C to 95 °C, with 1.9 °C per 1 min increments to determine the PCR product specificity. The amplicons were visualized using 1% agarose gel electrophoresis, as previously described.

The library was prepared from RNA samples (D, E, F) using TruSeq Stranded Total RNA with a Ribo-Zero Plant Kit (Illumina; San Diego, CA, USA). The quantity and quality of the constructed library was analyzed using light cycle qPCR and TapeStation D1000 ScreenTape assay (Agilent; Santa Clara, CA, USA) for NGS (Illumina; San Diego, CA, USA) by an external provider (Macrogen; Seoul, Korea), following the manufacturer's protocols.

## Results and Discussion

### *Evaluation of available RNA extraction methods*

Prior to the development of the optimized method, several protocols used for extracting RNA from sunflower seeds either failed or yielded RNA samples with a low concentration and purity. The RNA extractions with the commercially available TRIzol™ and Plant RNA reagents were both unsuccessful because a pellet was not obtained; the latter also failed to produce distinct RNA bands (Fig. 1C, L16–17). The classic CTAB protocol by Chang et al. (1993) and its various modifications (Xu et al., 2010; Footitt et al., 2018) similarly showed suboptimal results (Fig. 1A, L1–7). Likewise, the modified Li and Trick method A described by Osio et al. (2019) did not display distinct RNA bands (Fig. 1B, L10–11).

The modified Ma and Yang, and modified Li and Trick B methods described by Mornkham et al. (2013) were tested because these rapid protocols were recommended for oilseeds. Visual inspection of the extracted RNA based on electrophoresis revealed that the modified Li and Trick method B (Mornkham et al., 2013) produced better results (Fig. 1B, L8–9 and L12–13). The use of the TRIzol™ reagent as the second extraction buffer demonstrated promising output; thus the above-mentioned

conditions were modified and tested. Unfortunately, the addition of the TRIzol™ reagent as the second extraction buffer to the Li and Trick (2005) method still did not produce RNA of sufficiently high quality (Fig. 1C, L14–15). Several adjustments to the conditions of the Li and Trick (2005) protocol also failed to improve RNA purity (data not shown). Thus, further modifications were made to the previously published RNA extraction protocols.

*Optimized two-stage extraction method*

To develop an optimized method for high yields of high-quality RNA, extraction was performed in two stages. First, biological material was subjected to the original method by Li and Trick (2005) as modified by Osio et al. (2019). Second, treatment with the TRIzol™ reagent was performed to facilitate the extraction of high-quality RNA from sunflower seeds. The following modifications were introduced: 1) the amount of extraction buffer was increased from 400 µL to 800 µL per 100 mg of powdered seed; 2) the volume of the phenol-chloroform (1:1) mixture was increased from 250 µL to 500 µL; 3) TRIzol™ reagent was used to replace the second extraction buffer (70% guanidinium sulfate, w/v; 0.75 M sodium citrate; 10% N-laurylsarcosine; 2 M sodium acetate, pH 4.0); 4) RNA recovery by precipitation with an equal volume of isopropanol was performed for 15 min at -20 °C instead of on ice; and 5) the amount of 70% ethanol for washing the RNA pellets was increased from 400 µL to 800 µL. Intact and distinct RNA bands were observed after the above-mentioned protocol was performed (Fig. 1C, L18–19).

The optimized method was repeated for sunflower seed samples from the same lot to validate the results. DNase treatment after extraction was performed for samples contaminated with genomic DNA to obtain intact and sharp RNA bands with no smearing or signs of degradation (Fig. 2, L2–4). The entire extraction process could be completed in approximately 2 h, followed by 2 h of DNase treatment.

*RNA yield, purity and integrity assessment*

The extraction protocol efficiently produced high-quality RNA from samples A, B and C. Table 1 shows that the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios were both ≥ 2.00, implying the absence of contamination by proteins, polysaccharides and polyphenols. These values were higher than the absorbance ratios observed using the protocols modified by Mornkham et al. (2013). The RNA yields of the three samples were also sufficiently high for downstream applications. Furthermore, the sharpness of the three RNA bands (28s, 18s and 5s) on agarose gels demonstrated the high integrity of the extracted RNA (Fig. 2).

**Table 1** Purity and concentration of total RNA extracted from sunflower seeds (samples A, B, C) measured using a NanoDrop 1000 spectrophotometer

| Sunflower seed | Absorbance ratio* | | Concentration (ng/µL)* |
|---|---|---|---|
| | 260/280 | 260/230 | |
| Sample A | 2.09 ± 0.02 | 1.98 ± 0.04 | 740.10 ± 11.73 |
| Sample B | 2.10 ± 0.01 | 2.35 ± 0.05 | 629.43 ± 1.27 |
| Sample C | 2.13 ± 0.01 | 2.21 ± 0.05 | 736.23 ± 17.25 |

*average of three technical replicates and standard deviation for every sample

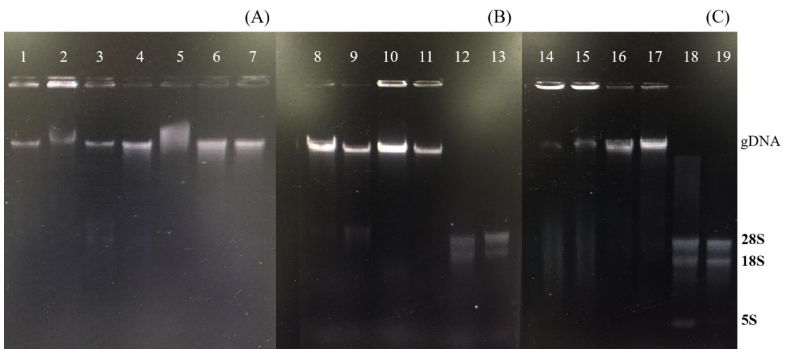


**Fig. 1** Gel electrophoresis of the total RNA samples from sunflower seeds using different extraction methods: (A) Lanes 1–7 = cetyltrimethyl ammonium bromide (CTAB) method with modified conditions (Chang et al., 1993); (B) lanes 8–9 = modified Ma and Yang method (Mornkham et al., 2013), lanes 10–11 = modified Li and Trick method A (Osio et al., 2019), lanes 12–13 = modified Li and Trick method B (Mornkham et al., 2013); (C) lanes 14–15 = Li and Trick method combined with TRIzol™ reagent, lanes 16–17 = Plant RNA reagent, lanes 18–19 = optimized two-stage method

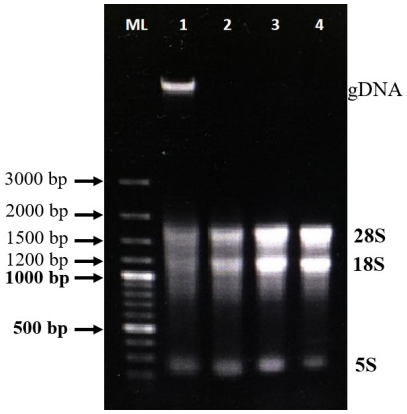


**Fig. 2** Gel electrophoresis of total RNA samples extracted from sunflower seeds using optimized two-stage method, where lane 1 = representative sample before DNAse treatment and lanes 2–4 = DNAse-treated samples (A, B, C, respectively)

Further analysis of the RNA quality of samples D, E and F using the 2100 Bioanalyzer produced RNA integrity numbers (RIN; 1 = poor quality, 10 = highest quality) in the range 6.4–8.2. The electrophoretograms of the three samples also showed distinct peaks and low levels of contaminants (Fig. 3).

### *Real-time reverse transcription-quantitative polymerase chain reaction and library preparation*

*Actin* is a housekeeping gene that has been used as an internal control for gene expression analysis (Ramu et al., 2016). The *actin* gene was amplified using RT-qPCR to assess the quality of the isolated RNA samples for subsequent molecular analysis. A standard curve was first generated using varying concentrations of the pooled RNA samples to determine PCR efficiency (Fig. 4). The RT-qPCR results and gel electrophoresis of amplicons are shown in Fig. 5 and Fig. 6, respectively. The expected amplification curves were produced (Fig. 5A) whereas the melting curves showed a single peak (Fig. 5C), thereby indicating the specificity of the amplified products.

Library preparation is the first step in the workflow of any NGS platform. Prior evaluation of the RNA quality and quantity is critical to avoid low performing libraries due to fragmented RNAs. For TruSeq Stranded Total RNA with a Ribo-Zero Plant Kit, an input RNA concentration of 10–200 ng/μL and an RIN value of ≥7 are required (Table 1 and Fig. 3). In addition, checking the concentration and size of the library will help to optimize cluster generation during RNA-seq. For most Illumina sequencing platforms, 5 nM (2 ng/μL) and 200 bp are the preferred starting library concentration and size, respectively (Table 2 and Fig.7). Hence, the extracted RNAs from the sunflower samples (D, E, F) were of sufficient quantity and quality for library construction.

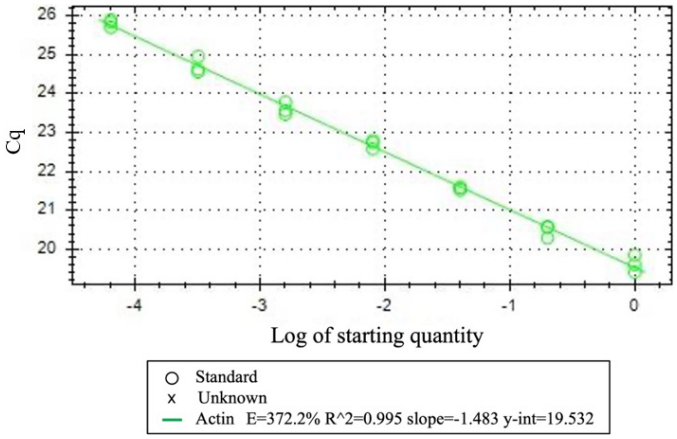


**Fig. 4** Standard curve for the *actin* gene confirming polymerase chain reaction amplification efficiency, where E = efficiency, R^2 = coefficient of determination and y-int = y intercept

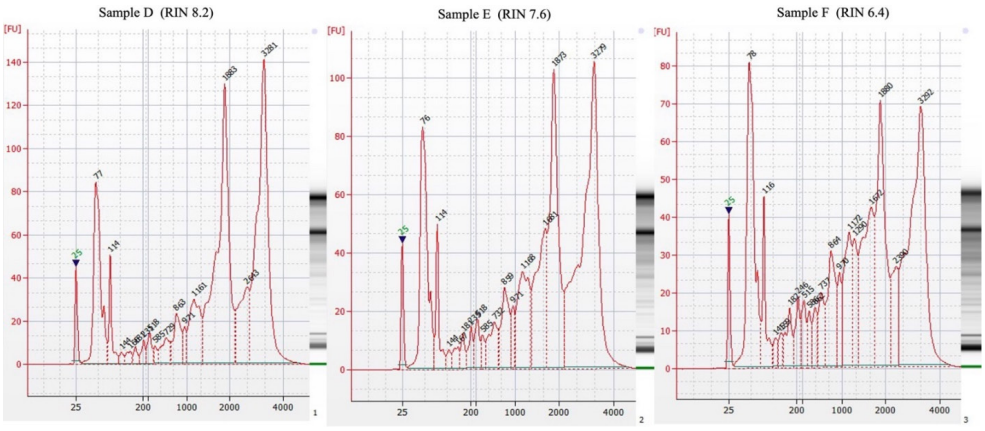


**Fig. 3** Gel electrophoresis and RNA integrity number (RIN) of total RNA samples extracted from sunflower seeds (samples D, E, F) analyzed using RNA Pico 6000 Chip Kit and Agilent 2100 Bioanalyzer

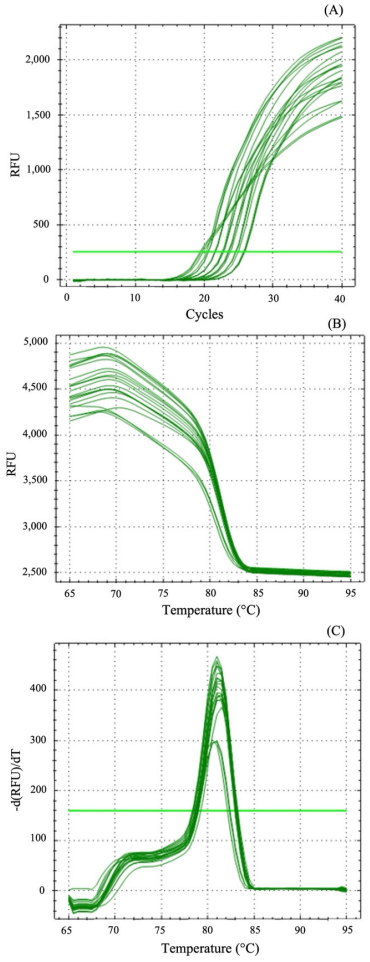


**Fig. 5** (A) Amplification curves; (B) melt curves; (C) melt peaks of *actin* gene as expressed in sunflower seeds, where RFU = relative fluorescence unit and -d(RFU)/dT = negative first derivatives of RFU data over temperature

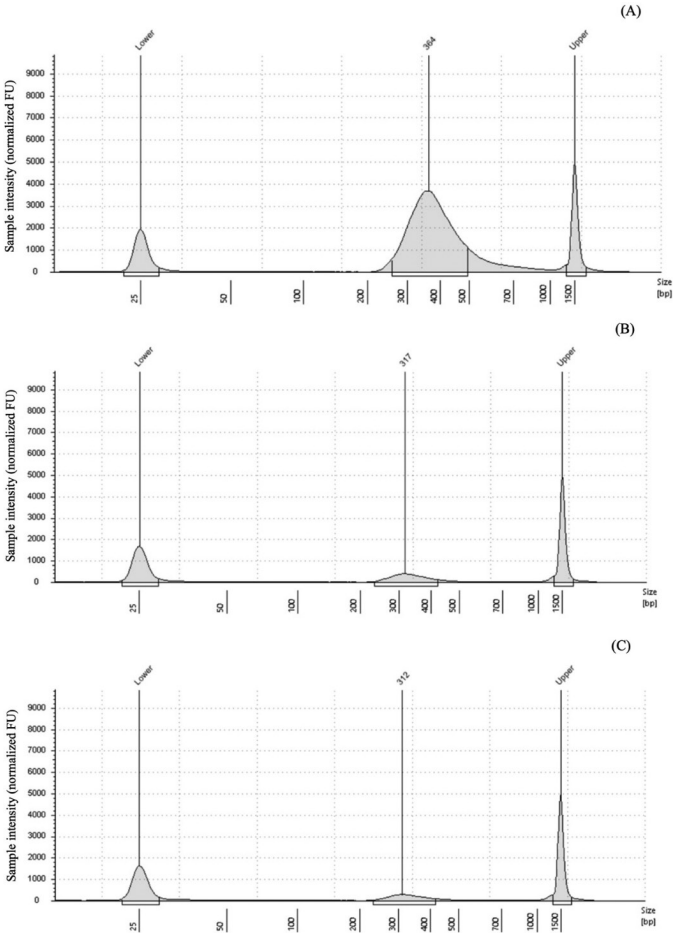


**Fig. 7** cDNA library constructed using TruSeq Stranded Total RNA with Ribo-Zero Plant Kit and analyzed via TapeStation D1000 ScreenTape assay for next-generation sequencing: (A) sample D (364 bp); (B) sample E (317 bp); (C) sample F (312 bp), where FU = fluorescence unit

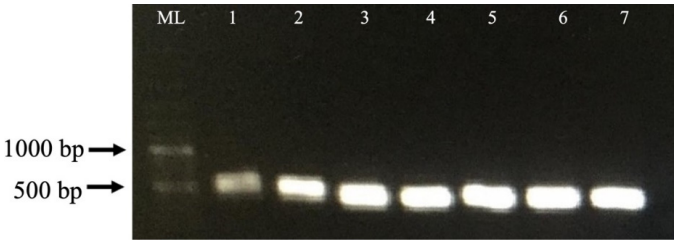


**Fig. 6** Gel electrophoresis of real-time reverse transcription-quantitative polymerase chain reaction-amplified cDNAs from sunflower seeds (samples A, B, C) using *actin* gene (lanes 1–7), where ML= molecular weight ladder

**Table 2** Concentration (Conc.) and size of library prepared from total RNA of sunflower seeds (samples D, E, F) analyzed using light cycle quantitative polymerase chain reaction

| Library name | Library type | Conc. (ng/μL) | Conc. (nM) | Size (bp) | Result |
|---|---|---|---|---|---|
| Sample D | TruSeq Stranded Total RNA with Ribo-Zero Plant Kit | 61.34 | 259.25 | 364 | Pass |
| Sample E | TruSeq Stranded Total RNA with Ribo-Zero Plant Kit | 5.74 | 27.85 | 317 | Pass |
| Sample F | TruSeq Stranded Total RNA with Ribo-Zero Plant Kit | 4.56 | 22.50 | 312 | Pass |

Pass = quality of library constructed is within the standards required for RNA-seq analysis using the Illumina platform

In conclusion, the current study demonstrated that the optimized method for sunflower seeds allowed rapid, high-throughput and economical extraction of high-quality and high-yield RNA. The efficiency of the extraction method was confirmed by the relative quantification of the selected gene and quality control analysis for NGS library construction.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

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## References

Badouin, H., Gouzy, J., Grassa, C.J., et al. 2017. The sunflower genome provides insights into oil metabolism, flowering and asterid evolution. Nature 546: 148–152. doi.org/10.1038/nature22380

Birtic, S., Kranner, I. 2006. Isolation of high-quality RNA from polyphenol-, polysaccharide-, and lipid-rich seeds. Phytochem Anal. 17: 144–148. doi.org/10.1002/pca.903

Chang, S., Puryear, J., Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. 11: 113–116. doi.org/10.1007/BF02670468

Chowdhury, H.A., Bhattacharyya, D.K., Kalita, J.K. 2020. Differential expression analysis of RNA-seq reads: Overview, taxonomy and tools. IEEE/ACM Trans. Comput. Biol. Bioinform. 17: 566–586. doi: 10.1109/TCBB.2018.2873010

Footitt, S., Awan, S., Finch-Savage, W.E. 2018. An improved method for the rapid isolation of RNA from *Arabidopsis* and seeds of other species high in polyphenol and polysaccharides. Seed Sci. Res. 28: 360–364. doi.org/10.1017/S0960258518000296

Li, Z., Trick, H.N. 2005. Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch. Biotechniques 38: 872–876. doi.org/10.2144/05386BM05

Ma, X.B., Yang, J. 2011. An optimized preparation method to obtain high-quality RNA from dry sunflower seeds. Genet. Mol. Res. 10: 160–168. doi: 10.4238/vol10-1gmr979

Mornkham, T., Wangsomnuk, P.P., Fu, Y.B., Wangsomnuk, P., Jogloy, S., Patanothai, A. 2013. Extractions of high-quality RNA from the seeds of Jerusalem artichoke and other plant species with high levels of starch and lipid. Plants 2: 302–316. doi.org/10.3390/plants 2020302

Nolan, T., Hands, R.E., Bustin, S.A. 2006. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 1: 1559–1582. doi.org/10.1038/nprot.2006.236

Osio, C.A.L., Aquino, G.M.B, Aala, W.F.Jr., Lado, J.P., Tendero, B.J.T., Cueto, C.A., Diaz, M.G.Q., Laude, R.P. 2019. Transcriptome analysis of 'Philippine Lono Tall' coconut (*Cocos nucifera* L.) endosperm reveals differential expression of genes involved in oil biosynthesis. Philipp. Agric. Scientist. 102: 101–118.

Ramu, V.S., Paramanantham, A., Ramegowda, V., Mohan-Raju, B., Udayakumar, M., Senthil-Kumar, M. 2016. Transcriptome analysis of sunflower genotypes with contrasting oxidative Stress tolerance reveals individual- and combined- biotic and abiotic stress tolerance mechanisms. PLoS One 11: e0157522. doi.org/10.1371/journal.pone.0157522

Rayani, A., Nayeri, F.D. 2015. An improved method for extraction of high-quality total RNA from oil seeds. Biotechnol. Lett. 37: 927–933. doi.org/10.1007/s10529-014-1752-6

Rocha, C.R.M., Silva, V.N., Cicero, S.M. 2014. Internal morphology and germination of sunflower seeds. J Seed Sci. 36: 48–53.

Salunkhe, D.K., Chavan, J.K., Adsule, R.N., Kadam, S.S. 1992. World Oilseeds: Chemistry, Technology and Utilization. Van Nostrand Reinhold. New York, NY, USA.

Salzman, R.A., Fujita, T., Zhu-Salzman, K., Hasegawa, P.M., Bressan, R.A. 1999. An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol. Biol. Rep. 17: 11–17. doi.org/10.1023/A:1007520314478

Wolf, B.B.W. 2013. Principles of transcriptome analysis and gene expression quantification: An RNA-seq tutorial. Mol. Ecol. Resour. 13: 559–572. doi.org/10.1111/1755-0998.12109

Xu, J., Aileni, M., Abbagani, S., Zhang, P. 2010. A reliable and efficient method for total RNA isolation from various members of spurge family (Euphorbiaceae). Phytochem. Anal. 21: 395–398. doi.org/10.1002/pca.1205

Zhang, H., Hu, Z., Yang, Y., et al. 2021. Transcriptome profiling reveals the spatial-temporal dynamics of gene expression essential for soybean seed development. BMC Genom. 22: 453. doi.org/10.1186/s12864-021-07783-z