Laboratory Exercise

RNA Isolation from Plant Tissues: A Hands-on Laboratory Experimental Experience for Undergraduates*

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ABSTRACT

The practice of RNA isolation in undergraduate experimental courses is rare because of the existence of robust, ubiquitous and stable ribonucleases. We reported here modifications to our original protocol for RNA isolation from plant tissues, including the recovery of nucleic acids by ethanol precipitation at 0 °C for 10 min and the assessment of RNA quality by visualizing the banding profile of the separated RNAs on a standard nondenaturing agarose gel to shorten the duration of the whole procedure and simplify the operation. As a result, the modified procedure, including RNA isolation and quality control analysis could be finished in 4 hr and divided into two sessions. Because endogenous ribonucleases released upon disruption of the organelles and vacuoles were effectively and quickly inactivated, measures were taken to protect RNA integrity throughout the whole procedure so that total RNA with high purity and integrity as well as an appropriate yield could be obtained by students. The RNA isolation protocol described here was simple, efficient, flexible, and low cost. Therefore, it is an ideal approach for undergraduates to learn about RNA techniques. The pedagogical approach of the correlation of experimental work with the rationale for the whole protocol described in this report is an effective way for undergraduates to improve their learning of the techniques of RNA isolation and analysis and the theories behind them, as well as experimental design and data analysis. © 2017 by The International Union of Biochemistry and Molecular Biology, 46(3):253–261, 2018.

Keywords: *RNA isolation; RNA quality control analysis; ribonucleases; undergraduate experimental course; laboratory exercises*

Introduction

Isolation of high-quality RNA is the primary requisite for subsequent studies in molecular biology. However, RNA isolation is considered to require too much expertise to be carried out by novices because ribonucleases (RNases) are very active, widespread, stable, and no-cofactor required. Therefore, only a few attempts at designing practical experiments for RNA isolation for undergraduates have been reported [1]. In some very recent reports about the study of gene expression in undergraduate experimental courses, RNA was prepared by commercial kits (e.g., [2–4]), and the kits are now also welcomed in research because of increased productivity, saved time and reduced costs [5]. However, we believe that working with kits is not the best choice for undergraduates to learn about RNA techniques. Previously, we reported an efficient and economic RNA isolation method based on phenol/chloroform extraction and selective fractionation of RNA by ethanol/sodium acetate (pH 5.6) precipitation [6]. In this method, endogenous RNases released during cell lysis are effectively and quickly inactivated, and measures are taken to protect the integrity of the RNA. Because no special reagents or equipment or complicated techniques are required, our method could be performed in a routine plant molecular biology lab with reproducible results by even novice investigators. However, our original protocol was unsuitable for an undergraduate experimental course because of the long duration (approximately 8 hr for only the isolation procedure) and because

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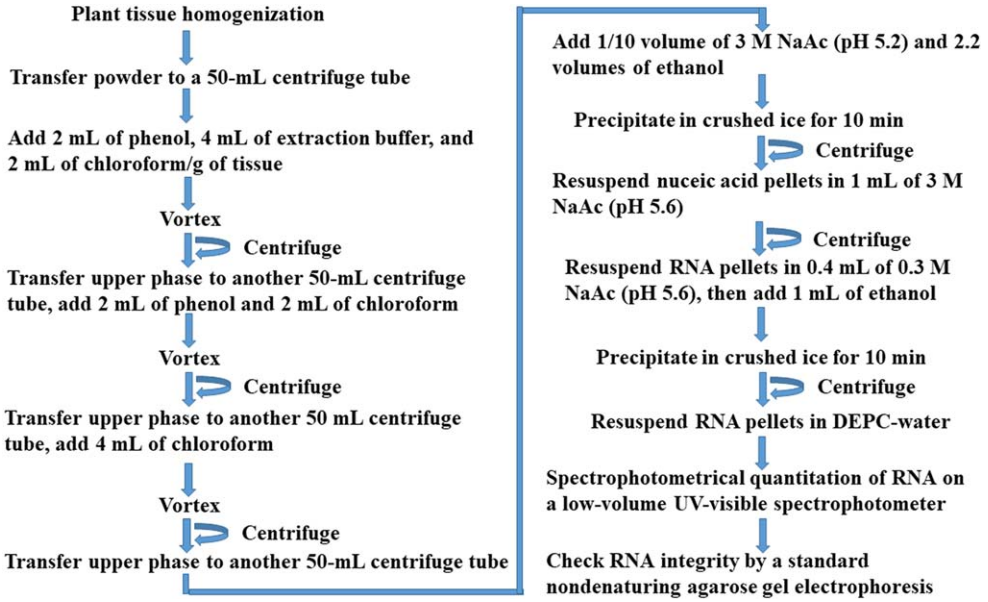

FIG 1 *Flowchart for purification of RNA from Chinese cabbage leaves and subsequent RNA quality control analysis. [Color figure can be viewed at wileyonlinelibrary.com]*

of the two long intervals of ethanol precipitation at −20 °C for 3 hr that were required to recovery nucleic acids [6]. Furthermore, the traditional method of assessing RNA integrity by visualizing the banding profile of the size-separated RNA by electrophoresis through agarose gels under denaturation conditions [5, 7, 8], is also unsuitable for an undergraduate experimental course because of the use of toxic denaturants and the complicated procedure. In this report, we modified our original protocol [6] to make it more practical for an undergraduate experimental course (Fig. 1). First, the long low temperature incubation for ethanol precipitation of nucleic acids [6] was replaced by precipitation at 0 °C for 10 min [9]. To achieve good recovery, the centrifugation times following ethanol precipitation were all set to 15 min. Second, the integrity of the RNA was determined by minigel electrophoresis of RNAs for low amount (less than 5 μg) using a standard, nondenaturing 1.5% agarose gel. Furthermore, in addition to the purity estimation by ratios of A_{260}/A_{280} and A_{260}/A_{230}, the ultraviolet (UV) spectrum analysis from 220 to 350 nm was introduced to help undergraduates better understand the UV absorption property of RNA and assess potential contaminants within the samples by observing deviations from the standard shape of the absorbance profile. The modified procedure (Fig. 1) could be finished in 4 hr but can also be divided into two sessions. In addition to being efficient and having a low cost like our original protocol [6], the modified procedure was simple and easy to operate and arrange, which makes it suitable for an experimental course for relatively unskilled undergraduates. Our method follows a typical RNA extraction procedure with cell lysis, the release and dissociation of nucleoproteins, denaturation and removal of proteins, precipitation of nucleic acids, removal of DNA and other impurities (Fig. 1). Therefore, it is truly representative of RNA isolation procedures in RNA research. Hands-on laboratory experience of RNA isolation and subsequent quality control analysis could help undergraduates learn basic techniques and the rationale that is important in RNA isolation and analysis as well as the difficulties and precautions involved in handling RNA. The correlation of the experimental work with relevant underlying concepts could help students learn how to design an experimental protocol based on the key and the goal of the experiment. An assessment of the efficacy of the pedagogical approach described in this report demonstrated that our experimental exercises were effective for enhancing student learning of the experimental skills in RNA research and relevant underlying concepts as well as the thinking skills required for conducting research.

Experimental Procedures

Theoretical Background

The source of contamination by RNases during RNA extraction can be exogenous or endogenous [5–7]. Exogenous sources include the reagents, glassware, and plasticware used in RNA isolation, especially the skin of the investigator. However, these RNases can be eliminated through sensible measures, such as treatment of reagents and plastic utensils with diethyl pyrocarbonate (DEPC); baking the glassware, mortar and pestle; and wearing disposable gloves throughout the whole procedure. However, endogenous RNases are innate to biological tissues and are normally sequestered in organelles and vacuoles. They are

highly regulated in intact cells, and the regulatory mechanisms are destroyed once the organelles and vacuoles are disrupted during cell lysis, which could lead to rapid degradation of RNA [5]. Therefore, how to quickly and completely inactivate the released endogenous RNases is the key to successful purification of high-quality RNA. Guanidinium salts have been shown to be powerful inhibitors of RNases [5, 7] and many guanidinium salt-based methods have been established for RNA isolation. However, some drawbacks still exist for these methods, such as RNA loss or fragmentation during organic extraction, interference of downstream enzymatic reactions by guanidinium salt residue, and a higher cost for the experiment because of the using of a higher concentration of guanidinium salts to inactivate RNases [7]. In our previously reported RNA isolation method [6], we tried to quickly and completely inactivate endogenous RNases released during cell lysis as well as overcome some drawbacks of guanidinium salt-based methods. Our measures included modifying the composition of the extraction buffer: the pH of the extraction buffer was not adjusted with HCl, and the final pH, including 0.2 M Tris, in our system is 9.0. This pH could greatly reduce RNase activity because only less than 15% of the activity remains when the pH is above 9.0 compared with the maximum activity at a pH of approximately 7.2 in water [10]. In addition, $MgCl_2$ and sucrose were included in the extraction buffer. The $MgCl_2$ was added because Mg^{2+} are needed to stabilize many secondary and tertiary structures within the RNA [11], and the sucrose was added to maintain an appropriate osmotic pressure for the solution. Otherwise in hypotonic solution, the fragmentation of RNA might occur through impingement of the osmotic pressure on RNAs on the explosive cell lysis. Furthermore, Tris-saturated phenol was added immediately into liquid nitrogen-ground tissue powders to inactivate the released endogenous RNases. During the following organic extraction, vortexing was adopted to enhance the effects of protein denaturation. Inclusion of $MgCl_2$ and sucrose in extraction buffer could also avoid the potential threat of fragmentation of RNAs, especially those with high molecular weight by vortexing. To reduce RNase activity introduced accidently during the whole isolation procedure, all reagents used for RNA preparation were ice-cold, the tubes were prechilled, and the samples were maintained on ice at all times, except during steps for air drying the nucleic acid sediments after centrifugation [5].

Nucleic acid concentration is usually the final step in most RNA purification protocols. Normally, the RNA concentration is achieved by precipitation in the presence of sodium ions and ethanol. However, unlike the dramatic precipitation of genomic DNA, longer incubation periods at −20 °C are often required for RNA precipitation to ensure complete recovery [5]. In our original protocol, nucleic acid recovery was achieved through ethanol precipitation at −20 °C for 3 hr, and included two ethanol precipitation steps, with one to precipitate nucleic acids, including both RNA and DNA, and the other to selectively precipitate RNA [6]. The whole isolation procedure lasted for approximately 8 hr and was divided into three sessions by two longer ethanol precipitations, which makes the method unsuitable for time-limited undergraduate experimental course. There is evidence showing that the recovery of nucleic acids by ethanol precipitation is not significantly enhanced by long or low temperature incubation, but by longer centrifugation time [9]. Therefore, the longer low temperature incubation (at −20 °C for 3 hr) was replaced by incubation at 0 °C for 10 min, and the centrifugation time following ethanol precipitation were all set to 15 min to achieve good recovery. As a result, the duration of the isolation procedure was substantially reduced (in 2.5 hr, compared to 8 hr of our original method), while the recovery of RNA was comparable to our original method (data not shown).

Because the success of many downstream RNA-based applications is relied on obtaining high-quality RNA, it is necessary to assess the quality of purified RNA before conducting downstream assays. Therefore, the quantity, purity, and integrity of RNA samples should be checked with appropriate methods. RNA has a maximum absorption at 260 nm, and the RNA concentration could be quantified spectrophotometrically by the relationship 1 $A_{260} = 40$ μg RNA mL^{-1}. Contaminated proteins and polysaccharides/polyphenols have maximum absorption values at 280 and 230 nm, respectively, so the ratios of A_{260}/A_{280} and A_{260}/A_{230} could be used as indications of these contaminants. Additionally, these contaminants could result in deviations from the standard absorbance spectrum with the characteristic UV absorption profile of pure RNA samples, which is not observable when only measuring absorbance at the three wavelengths above. Therefore, besides absorption ratios, the UV absorbance spectrum analysis of purified RNA from 220 to 350 nm was introduced to assess the purity of RNA. To check RNA integrity, the typical method is to visualize the banding profile of size-separated RNA bands through an agarose gel under denaturing conditions. However, it is a time-consuming process because of the complicated procedure, and the visualization of RNA bands cannot be achieved immediately after electrophoresis either [8]. A standard agarose gel, although it has lower resolution for nondenatured RNAs rich in secondary and tertiary structures, has the advantages of being easy to complete and capable of visualizing RNA immediately after electrophoresis by including ethidium bromide (EtBr) in the gel. Therefore, electrophoresis of nondenatured RNA through a standard agarose gel was used to check RNA integrity in this report. However, it should be noted that the best performance of the separation of total RNA under nondenaturing conditions could only be achieved by running RNA samples with lower amounts (usually less than 5 μg) through a higher concentration of agarose gel (usually 1.5%).

Arrangement

Students worked in pairs in the lab. The RNase-free treatment was performed before the experiment by individual students. Approximately 1 hr of prelab lecture and discussion focused on specific precautions for working with RNA, and the rationale for establishing the original protocol and the modifications made for the methods of RNA isolation and RNA quality control analysis used in this report were provided and followed by a 4 hr hands-on lab exercise. The whole procedure could also be divided into two sessions of RNA isolation and RNA quality control analysis.

Materials and Solutions

The following equipment is required for the experiment: mortar and pestle, stainless lab spatulas, 50-mL centrifuge tubes, pipettes, vortex, disposable tips and gloves, centrifuge (Thermo Fisher Scientific, Am Kalkberg, Germany, Sorvall ST 16R), 1.5 mL Eppendorf tubes, low-volume spectrophotometer (NanoDrop Technologies, Wilmington, DE, Nanodrop 2000c), submarine electrophoresis facilities (Bio-Rad, Hercules, CA, Sub Cell GT), and gel imaging system (Bio-Rad, Hercules, CA, Gel Doc XR system). The RNase-free treatment was performed as follows: all tubes and tips were soaked in 0.1% DEPC overnight at 37 °C, then autoclaved at 121 °C for 20 min. The mortars and pestles as well as the stainless lab spatulas were baked at 180 °C for 12 hr.

The following reagents were required for RNA isolation: RNA extraction buffer (0.2 M Tris, 0.4 M KCl, 0.2 M sucrose, 35 mM $MgCl_2$, 25 mM EGTA), Tris-saturated phenol (pH 8.0), chloroform/isoamyl alcohol (24:1, v/v), 3 M NaAc (pH 5.2), 3 M NaAc (pH 5.6), 0.3 M NaAc (pH 5.6), and DEPC-treated water. NaAc solutions and DEPC-treated water were prepared by treating NaAc solutions and water with 0.1% DEPC overnight at 37 °C, and then autoclaved at 121 °C for 20 min. The RNA extraction buffer was prepared with DEPC-treated water.

The following reagents were required for RNA analysis: agarose, 1 × TAE, 10 × RNA loading buffer (1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol), and 10 mg/mL EtBr. Solutions for RNA electrophoresis were not administered the RNase-free treatment because RNase and RNA bands have different mobility in agarose gels, which means they could be separated once an electric field is applied.

Cell Lysis, Nucleoprotein Dissociation, Protein Denaturation, and Removal

The following procedure can be applied to a wide variety of herbaceous plant tissues. For plant tissues rich in polysaccharides and/or polyphenols, specific purification protocols should be adopted, such as the modified cetyltrimethylammonium bromide method [12].

Collect 1 g of Chinese white cabbage (*Brassica campestris* L. ssp. *chinensis* Makino [var. *communis* Tsen et Lee]) leaves and grind them in liquid nitrogen in a precooled mortar to a fine powder. Do not let the tissue thaw and immediately transfer the powder to a 50-mL centrifuge tube, add 2 mL of Tris-saturated phenol (pH 8.0) immediately, then 4 mL of extraction buffer and 2 mL of chloroform/isoamyl alcohol (24:1, v/v) sequentially (phenol should be added first to establish a denaturation environment for the endogenous RNases to be released into). Vortex the tube until a complete emulsion was formed. Centrifuge at 8,000 × *g* for 5 min at 4 °C. Carefully transfer aqueous phase to another 50-mL centrifuge tube, then add 2 mL of phenol and 2 mL of chloroform/isoamyl alcohol (24:1, v/v), mix vigorously and centrifuge at 8,000 × *g* for 5 min at 4 °C. Take the upper phase and add 4 mL of chloroform/isoamyl alcohol (24:1, v/v), then mix and centrifuge the sample as before. Transfer the upper phase to a 50-mL centrifuge tube and record the volume that was transferred.

Selective RNA Precipitation

Combinations of ethanol and NaAc (pH 5.6) were used to selectively precipitate the RNA. First, add 0.1 volume of NaAc (pH 5.2) and 2.2 volumes of ethanol precooled at −20 °C to the transferred aqueous phase and mix it by inversion several times. After embedding the tubes in crushed ice for 10 min, centrifuge them at 15,000 × *g* for 15 min at 4 °C. The nucleic acids were spun onto the wall of the centrifuge tube. After centrifugation, decant the liquid and mark the location of nucleic acid sediments on the outer wall of the tube. Invert the tube and place it on a filer paper to allow it to air-dry to remove residual ethanol. Resuspend the nucleic acid sediments with 1 mL of 3 M NaAc (pH 5.6) using a pipette, then transfer the resuspended nucleic acid to a 1.5 mL Eppendorf tube. Centrifuge the tube at 15,000 × *g* for 10 min at 4 °C, carefully decant and discard the supernatant, place the tube invertedly on a filter paper to air dry the nucleic acids. Redissolve the sediment in 400 μL of 0.3 M NaAc (pH 5.6) and add 1 mL of ethanol precooled at −20 °C, mix the tube by inversion several times, embed the tube in crushed ice for 10 min, then centrifuge the tube at 15,000 × *g* for 15 min at 4 °C. Wash the RNA pellet twice with 200 μL of 70% ethanol, then air-dry and dissolve the pellet in 50 μL of DEPC-treated water.

RNA Quality Assessment by Spectrophotometer

The RNA was analyzed on a NanoDrop 2000c spectrophotometer according to the manufacturer's instructions. An absorbance spectrum was obtained from 220 to 350 nm, the RNA concentration was calculated with the equation 1 $A_{260} = 40$ μg RNA mL^{-1}, and ratios of A_{260}/A_{280} and A_{260}/A_{230} were calculated to evaluate the purity of the RNA samples that were extracted.

Agarose Gel Electrophoresis

Dilute 10 μL of RNA samples to 1 μg/μL with DEPC-treated water according to the quantification results, 0.5, 1, 2, and 4 μg of RNA aliquots were taken out and adjusted to 9 μL with DEPC-treated water, then 1 μL of 10 × RNA loading

buffer was added. After mixing the samples, all aliquots were loaded onto a 1.5% TAE agarose gel containing 0.5 μg/mL EtBr. Electrophoresis was carried out in 1 × TAE at 5 V/cm until the dye front had migrated two-thirds of the way down the gel. The gel was photographed with a Gel Doc XR System (Bio-Rad).

Assessment of Student Learning

Three methods were applied to assess student learning from the experimental exercises: Lab reports after the experiment, a lab presentation at the beginning of the next experimental class, and a short summary after the whole experimental course. In their lab reports, students were required to state the purpose and the principles of the experiment, concisely state the results that were obtained, including the yield in μg RNA/g of fresh weight of the leaves, the ratios of OD_{260}/OD_{280} and OD_{260}/OD_{230}, and the results of the electrophoresis of nondenatured RNAs in a standard agarose gel in a figure, as well as state the key of the protocol design and the experimental operation, especially how to prevent RNase contamination, and state what conclusions they can make based on their data. In addition, six groups out of 15 were randomly selected to give a presentation before the whole class about their results and experiment. Thus, all of the groups could mutually compare their results and, if necessary, discuss their experimental experience. In their summary, they should list what they had learned, including their grasp of experimental skills and reinforcement of the fundamental concepts.

Hazards

The liquid nitrogen used for freezing plant tissues needs to be handled carefully. Phenol and chloroform can easily evaporate, even at room temperature, and these two reagents as well as their vapors are corrosive to the eyes, the skin, and the respiratory tract. EtBr is mutagenic and should be used with caution. Phenol, chloroform, and DEPC are carcinogenic and should be handled with extreme care. Students were required to wear lab coats and closed-toed shoes in the lab as well as disposable gloves throughout the whole procedure.

Results and Discussion

RNA isolation is a process that is considered to require too much expertise to be carried out by undergraduates. However, as an important biomacromolecule, it would be imperfect for undergraduates if they have no experience conducting experiments with RNA manipulations. RNA isolation is a basic RNA manipulation technique and is the basis of other RNA manipulations. However, the methods used in molecular biological research cannot be directly applied for this purpose because either special equipment or reagents are required, or they are technically complicated, or have a long duration. In our previously reported RNA isolation method [6], the composition of the extraction buffer was modified to quickly and completely inactivate the endogenous RNases that are released during cell lysis, and measures were taken to maintain RNA integrity. In this report, we mainly modified the conditions of ethanol precipitation of nucleic acids, and the modified protocol included precipitation with ethanol by incubation tubes in crushed ice (0 °C) for 10 min, then the tubes were centrifuged for 15 min to collect the insoluble nucleic acids. As a result, the duration of RNA isolation was shortened to 2.5 hr. Furthermore, we also modified the quality control methods: the agarose gel electrophoresis for checking RNA integrity was performed by directly running nondenatured RNAs on a standard agarose gel; and besides UV absorbance measurement, a spectrophotometric spectrum analysis was introduced for checking RNA purity and assessing the presence of possible contaminants in RNAs. The duration for RNA quality control measurements was 1.5 hr. The whole procedure required 4 hr to be carried out, which is only about half of the duration of a previous reported protocol [1] and our original protocol [6], but can be divided into two sessions, which made the modified method easy to arrange for an undergraduate experimental course.

TABLE I ***Quantity and quality of total RNA isolated from Chinese white cabbage leaves***

Group ID	*Yield (μg/g fresh weight)*	*OD_{260}/OD_{280}*	*OD_{260}/OD_{230}*
1	247.8	1.94	2.23
2	531.6	1.91	2.37
3	425.0	1.98	2.09
4	317.3	2.01	2.12
5	337.4	1.92	2.37
6	412.1	2.03	2.02
7	216.9	1.93	2.27
8	595.4	1.93	2.41
9	378.3	2.04	2.33
10	289.2	1.93	2.21
11	231.5	2.06	2.34
12	246.7	2.03	2.05
13	321.6	1.97	2.32
14	427.5	1.93	2.28
15	281.7	2.04	2.15

Analyzed by a NanoDrop 2000c.

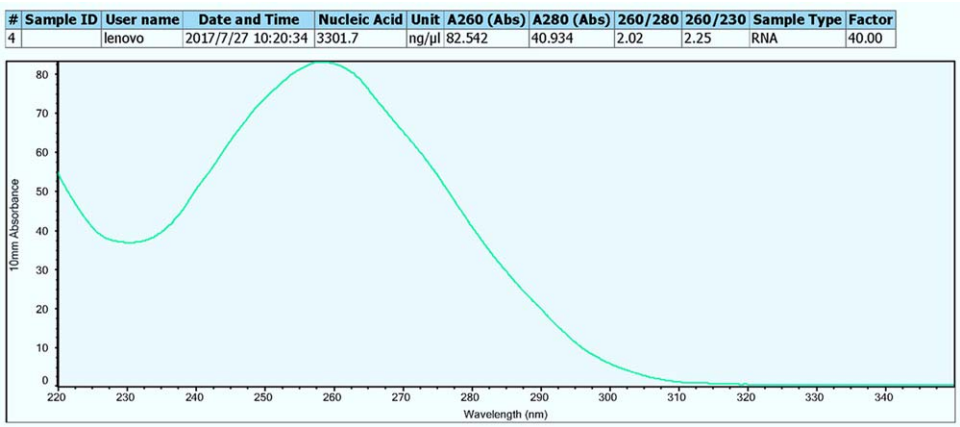

FIG 2 *The UV absorbance spectrum of purified RNA measured by a NanoDrop 2000c spectrophotometer. The absorption spectra of total RNA extracted from Chinese cabbage leaves were measured at wavelengths from 220 to 350 nm. [Color figure can be viewed at wileyonlinelibrary.com]*

Because no special reagents, equipment, or techniques were required, our method was simple and easy to grasp as well as suitable for novice investigators without too much training in molecular biology techniques. Table I shows a representative result from a class in 2013 for experimental course practice. The ratios of OD_{260}/OD_{280} and OD_{260}/OD_{230} were 1.9–2.1 and 2.0–2.4. Because a pure sample of RNA has the ratios of A_{260}/A_{280} and A_{260}/A_{230} just in this range [5], these results indicated that the RNA had good purity and integrity even though it was prepared by undergraduates. However, there were variations in the yield that ranged from 200 to 600 μg of RNA/g fresh weight with an average of 350 μg of RNA/g fresh weight. The spectrophotometric spectrum analysis from 220 to 350 nm (Fig. 2) shows the plot characteristic of an absorption peak near 260 nm and an absorption valley at 230 nm without any shoulder peaks, which further indicated that the RNA isolated had high purity. A representative result of the separation of nondenatured RNA through a standard 1.5% agarose-TAE gel (Fig. 3) showed several distinct rRNA bands without smears, and the brightness of 25S rRNA was about two times higher than the 18S rRNA. Because of the relatively low resolution of the standard agarose gel for nondenatured RNAs, the banding profile indicated that the RNA isolated had high integrity. Furthermore, little, if any, genomic DNA contaminated the RNA samples because there were no bands at a higher molecular weight on the gel (Fig. 3).

Because nearly all of the students had little, if any, training for hands-on experiments with RNA isolation, they completed the experiment with great care. Therefore, the big problem for them was not being careless, but being too careful. For example, they would perform inadequate transfer of the aqueous phase following centrifugation during organic extraction because of their fear that proteins might be carried over or perform only a gentle shake during organic extraction for fear that RNAs might be mechanically degraded. All of the possible problems that might be encountered, not only during an undergraduate experimental course, but in our research work, are listed

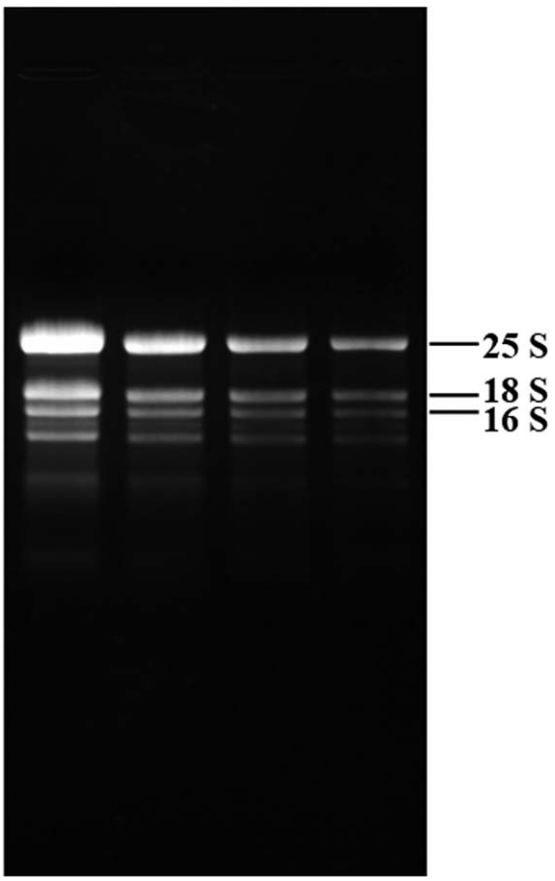

FIG 3 *Electrophoretic analysis of RNA. Total RNA was extracted from Chinese cabbage leaves and 0.5, 1, 2, and 4 μg of RNA were directly analyzed on a standard 1.5% agarose gel containing 0.5 μg/mL ethidium bromide.*

TABLE II *Troubleshooting table*

Problem	*Possible reason*	*Possible solution*
Nucleic acid pellets were hard to dissolve	Nucleic acid pellets dried out completely	Do not allow the pellet to dry out completely before dissolving. If this takes place, incubate the tube at 65?C for 10 min
Low yield	Old leaves were collected	Collect young leaves
	The homogenization was not enough	Homogenize the tissue in liquid nitrogen to a fine powder
	Low volume of aqueous phase transferred during organic extraction	Transfer adequate phase but avoid transferring denatured proteins at the interphase
	Not all nucleic acid pellets obtained from NaAc (pH 5.2)/ethanol precipitation were completely resuspended in 1 mL of 3 M NaAc (pH 5.6)	Flush more extensive area of pellets with NaAc (pH 5.6) and divide 1 mL of NaAc (pH 5.6) into two or three portions to flush
DNA contamination	The pH value of NaAc (pH 5.6) was not adjusted properly	Adjust pH value at 4?C
	The nucleic acid pellets were not completely resuspended in 1 mL of 3 M NaAc (pH 5.6)	Resuspend nucleic acid by drawing in and out with a disposable pipette tip
	The nucleic acid pellets were not completely dissolved in 0.4 mL of 0.3 M NaAc (pH 5.6) before adding 1 mL of ethanol	Make sure that the pellets completely dissolved before adding ethanol and see above nucleic acid pellets were hard to dissolve
Lower ratio of OD_{260}/OD_{280}	Proteins were not completely denatured during organic extraction	Denature proteins by vigorous shaking or vortexing during organic extraction; extract with phenol/chloroform once more, even a third time
	Proteins were carried over during the transfer of aqueous phase	Avoid disturbing the protein interface, leave a few microliters of aqueous material behind
Higher ratio of OD_{260}/OD_{280} (> 2.1)	RNA experienced more or less degradation. If the ratio was above 2.3, most of the RNA was degraded	Avoid thawing of the sample during grinding and transferring the ground powder to the centrifuge tube
		Operate with care once no sucrose or $MgCl_2$ included in the solutions
		Avoid contamination of RNase, including RNase-free treatment of solutions, glassware and plasticware, wear of clean gloves, use of prechilled reagents, maintenance of the sample on ice except for air-drying
Poor resolution of RNA bands in the gel	Lower concentration of agarose gel	Ensure higher concentration of agarose gel (1.5%) used to separate RNA
	Higher amount of RNA loaded	Requantfy RNA samples obtained
	Agarose was not melted completely	

TABLE II *(Continued)*

Problem	*Possible reason*	*Possible solution*
Smear or distorted RNA bands on the gel		Use longer heating time to dissolve higher concentration of agarose completely
	Water beads were on the surface of the teeth of the comb	Remove any water beads on the surface of the teeth of the comb
Brighter RNA bands at low molecular weight	Poor resolution of RNA bands	See above poor resolution of RNA bands
	RNA degraded	See above higher ratio of OD_{260}/OD_{280}
	Storage of RNA samples not in low temperature	Store RNA in low temperature, at least on the ice or better at −20 °C or −80 °C
	RNA degraded during electrophoresis if no RNase-free treatment of RNA electrophoresis solutions	Apply electric field immediately after loading, run the gel at slightly higher voltage of 5 V/cm

in Table II. Though these problems were rarely encountered in our undergraduate experimental course under the guidance of instructors, they were common issues for RNA isolation, so these problems, along with possible solutions, are also listed for reference.

It is equally important for undergraduates to grasp the rationale of the technique besides learning the technique itself in experimental courses. Here, we listed the rationale for establishing the original method and the modifications made in this report, which would be helpful for undergraduates to correlate the experimental work with relevant concepts about the chemistry of RNA and RNase as well as the theory of electrophoresis. Not only are fundamental concepts learned in their theory courses reinforced, but the principles of designing of an experimental procedure based on the key to a technique were also learned by students. For RNA purification, how to quickly and completely inactivate released endogenous RNases normally sequestered in organelles and vacuoles is just such a key. Furthermore, some basic principles should also be deliberately considered when designing and performing an experimental procedure. For example, the quantity and quality were a pair of contradictory values in any isolation and preparation procedure. In our method, the more aqueous phase that was transferred during organic extraction, the higher the RNA recovery was. However, this approach might result in more transfer of impurities, which could lower the purity of the RNA. The contradiction of the yield and purity should be seriously considered in an isolation experiment. Trade-off is not only merely a principle of designing experimental procedure but also an important way of thinking; when two or more protocols could be chosen for a specific purpose, determining which of the protocols should be adopted depends on the goal of the experiment. For example, agarose gel electrophoresis is a commonly used method for evaluating RNA integrity, and the electrophoresis could be carried out under denaturing or nondenaturing conditions. The standard nondenaturing agarose gel has poor resolution for nondenatured RNA. However, because of its simplicity and easy protocol, the risk of RNA degradation by RNases is greatly decreased, and the method is the best choice for undergraduates to check RNA integrity.

The feedback from students revealed that our introduction of the hands-on experiment of RNA isolation from plants was welcomed. A common feeling of the students after the experiment was “RNases are not as terrible as what others told me and I imagined”. In the lab reports and presentation, the students could analyze the data that were obtained and give reasonable explanations for the experimental results as well as problems they encountered. Additionally, they knew the key to the isolation of high-quality RNA and the corresponding way out. In the postlaboratory experiment summary, the students also noted what they gained not only at the research technical level, but also at the conceptual level and the improvements in

their way of thinking. Collectively, all of these outcomes indicated that the experimental exercises we introduced were ideal for the teaching and learning experimental techniques and relevant concepts as well as experimental design and data analysis skills.

In summary, high-quality RNA has been successfully isolated from plant tissues by undergraduates. The hands-on laboratory experience and the related background theory provide enrichment for students both the conceptual framework and the laboratory abilities. The rationale for establishing the previous method [6] and the modifications made in this report helps students comprehend the principles of the key- and the goal-based designing of the experimental protocol. Learning important techniques and scientific principles will be helpful for their future graduate programs or careers in biotechnology.

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