

# A rapid method for isolation of low-molecular-weight RNA from *Arabidopsis* using low salt concentration buffer

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## Abstract

We have developed a rapid extraction method using low salt concentration buffer for the isolation of RNA from *Arabidopsis* tissues. The method was quick and efficient, and the small scale extraction process took no more than one hour, while yield and RNA quality were comparable with those previously reported. The low-molecular-weight (LMW) RNA isolated using this method was high quality, abundant in small RNA and free of high molecular weight RNA. This method can be used to extract low-molecular-weight RNA for the purpose of small RNA cloning and detection, and library construction.

## Introduction

Low-molecular-weight (LMW) RNA, which includes 20- to 24-nt small interfering RNA and microRNA,<sup>2,3</sup> plays an important role in regulating gene expression by a mechanism known as RNAi (RNA interference or gene silencing). Though much attention has been devoted to an analysis of the functions of these small RNAs, an extraction protocol has not been optimized. Currently, small RNA extraction and detection methods fall into two major strategies. The first is to isolate total RNA and run on a polyacrylamide gel, then recover the small RNA from the gel or detect them using Northern blotting. However, this method needs to load large amounts of total RNA and often causes irregular banding due to overloading. Therefore, a preferred strategy is to use the LMW RNA for separating and detecting small RNA. The LMW RNA extraction protocol usually includes stepwise precipitation to eliminate DNA and high-molecular-weight (HMW) RNA, which makes the extraction process tedious and time-consuming. Besides, the lengthy extraction process is liable to cause RNA degradation.

Plant tissues are rich in polysaccharides,

polyphenols and other secondary metabolites which make the isolation of good-quality LMW RNA more difficult.<sup>4,7</sup> The secondary metabolites can interfere with nucleic acid separation and result in poor yield of LMW RNA. A cetyltrimethylammonium bromide (CTAB) based method combined with PVP could overcome this problem.<sup>8</sup> However, even this optimized protocol took no less than two days. Another choice is to use the commercial kits; but most of them are expensive. Therefore, there is the need for a quick and simple method to isolate LMW RNA.

Here we report a novel and efficient method for the extraction of LMW RNA from plant tissues, which is convenient and less time consuming than current methods. This method also adopts CTAB and polyvinyl pyrrolidone (PVP) to eliminate secondary metabolite contaminations. But the key point is using low salt concentration (LSC) extraction buffer containing 0.4 M sodium acetate. In the cells, DNA and HMW RNA are bound to proteins and form complexes termed DNP and RNP which do not dissolve in the low salt concentration buffer,<sup>9</sup> while the LMW RNA remains soluble. Thus, LMW RNA can be separated from DNA and HMW RNA during centrifuging and no extra eliminating steps were needed. Then the LMW RNA in the LSC buffer can be easily recovered. In this study, we used this method to extract LMW RNA from different tissues of *Arabidopsis* and obtained good results. In addition, the utility of the extracted LMW RNA was further investigated.

## Materials and Methods

### Plants

*Arabidopsis* plants (Col-0) were grown in a controlled growth chamber at 23±1°C with a 16 h light/8 h dark photoperiod. The plant samples were harvested at four weeks old and frozen immediately in liquid nitrogen.

### Solutions

#### LSC Extraction buffer

2% CTAB  
2% PVP  
0.4 M NaAC (pH 5.2)  
20 mM EDTA  
2% β-ME (add just before use)

#### Washing buffer (pH 7.4)

25 mM Tris.Cl  
10 mM EDTA  
80% ethanol

#### TE buffer (pH 8.0)

10 mM Tris  
1 mM EDTA

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Key words: low-molecular-weight RNA, low salt concentration buffer, *Arabidopsis*.

Contributions: HC, experimental design, sample collection, RNA extraction, Northern blotting; JG, RNA extraction; ZA, RNA PAGE; HH, experiment instruction, manuscript preparation.

Acknowledgments: we thank Dr. Tang Chaorong for helpful discussion. This work was supported by National Natural Science Foundation of China (30960319).

Conflict of interest: the authors report no conflicts of interest.

Received for publication: 23 January 2010.

Revision received: 26 July 2010.

Accepted for publication: 27 July 2010.

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International Journal of Plant Biology 2010; 1:e14  
doi:10.4081/pb.2010.e14

### PCI (Phenol-chloroform-isoamylalcohol, 25:24:1)

50 mL water saturated phenol  
48 mL chloroform  
2 mL isoamylalcohol  
Total volume 100 mL

### CI (chloroform-isoamylalcohol, 24:1)

96 mL chloroform  
4 mL isoamylalcohol  
Total volume 100 mL

### Small scale extraction

Step 1: grind 50 mg tissue into fine powder;  
Step 2: transfer the sample into 0.5 mL LSC extraction buffer and vortex vigorously for 1 min;  
Step 3: incubate at 65°C for 10 min with occasionally swirling;  
Step 4: centrifuge at 20,000g for 10 min at 4°C;  
Step 5: transfer the upper liquid phase (about 400 μL) to new tube;  
Step 6: add 200 μL of isopropanol and mix well;  
Step 7: transfer the mixture into an RNase free silica spin column (Tiangen, Beijing, China), and centrifuge for 30 sec at 6,500 g;  
Step 8: add 700 μL washing buffer and centrifuge for 30 sec at 6,500g;  
Step 9: discard the flow through and repeat step 8;  
Step 10: centrifuge for 1 min at 12,000 g;

Step 9: add 50  $\mu$ L TE buffer onto the silica membrane and elute the LMW RNA into a new RNase free tube.

#### Large scale extraction

Step 1: grind 1 g tissue into fine powder;

Step 2: transfer the sample into 10 mL LSC extraction buffer and incubate at 65°C for 10 min, then place the tube on ice;

Step 3: add 10 mL PCI, vortex vigorously for 1 min;

Step 4: centrifuge at 30,000g for 10 min at 4°C;

Step 5: transfer the supernatant into new tube;

Step 6: add 10 mL CI, vortex and centrifuge at 30,000 g for 10 min at 4°C;

Step 7: recover the supernatant, add 10 mL ice cooled isopropanol and incubate at -20°C for more than 1 h;

Step 8: centrifuge at 30,000 g for 20 min at 4°C, and discard the supernatant;

Step 9: re-suspend the precipitate with 0.5 mL LSC buffer and transfer to a 1.5 ml tube;

Step 10: extract the sample sequentially with 0.5 mL PCI and CI, and collect the supernatant;

Step 11: add 0.5 mL isopropanol and incubate at -20°C for more than 30 min;

Step 12: centrifuge at 20,000 g for 10 min at 4°C, and discard the supernatant;

Step 13: wash the precipitant twice with 80% ethanol, and dissolve in 50  $\mu$ L TE buffer.

#### Low-molecular-weight RNA polyacrylamide gel electrophoresis and Northern blotting

For LMW RNA electrophoresis, 3  $\mu$ g LMW RNA of each sample were denatured and loaded onto urea (8%) 15% polyacrylamide gels. After electrophoresis, the gels were stained with 1mg/L ethidium bromide (EB) for 30 min and visualized under ultraviolet light.

For Northern blotting, about 30 microgram LMW RNA was run on 15% denaturing polyacrylamide gel electrophoresis (PAGE) gel until the bromophenol blue band reached the bottom. Then the LMW RNA was transferred to nylon membrane using semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). The hybridization procedure was carried out as described,<sup>10</sup> except use of the 5'-digoxigenin labeled probe. After hybridization and 3 washes, the blot was detected using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Basel, Switzerland).

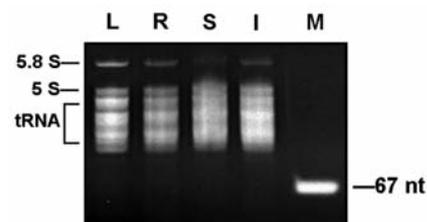
## Results and Discussion

LMW RNA was extracted from the leaf, root, stem and inflorescence tissues of *Arabidopsis*, and in each case high quality LMW RNA was obtained. Spectrophotometry was used for LMW RNA quantification and quality checking

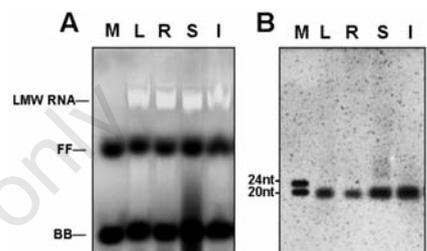
by calculating the A260/A230 and A260/A280 ratio. These ratio values fell into the range from 1.6 to 2.2 (*Supplementary Tables 1 and 2*), which were almost the same as those of previously reported studies<sup>8</sup> and indicated low polysaccharide and protein contamination. The yield of our method is about 50  $\mu$ g/g FW for *Arabidopsis* (*Supplementary Table 1*) and was marginally lower than that reported.<sup>8</sup> LMW RNA samples of all these tissues were subjected to 15% denaturing polyacrylamide gel electrophoresis (PAGE), and distinct 5.8S and 5S ribosomal bands were sharp and intense, indicating good recovery rate and intactness (Figure 1). In addition, no extra band existed above the LMW band (Figure 2A), indicating HMW RNA and DNA were eliminated. This conclusion was also proved by 2% agarose gel electrophoresis.

To check the downstream applicability of the LMW RNA extracted using this method, Northern blotting was carried out for the expression analysis of specific miRNA. Here we analyzed the expression of one *Arabidopsis* miRNA, AtMIR319C (miRNA sequence, 5'-UUUGGACUGAAGGGAGCUCC-3') which had been previously revealed to express at moderate levels in leaves, roots, and young seedling but strongly in stems and inflorescence.<sup>10</sup> After electrophoresis, the gel was stained with ethidium bromide and visualized. As shown in Figure 2A, a distinct smear appeared between the indicator bands of xylene cyanol FF and bromophenol blue, which was about the size of 20-60 nt. This range covered almost all of the known small RNA species. MIR319C expression was detected in all LMW RNA samples, and was particularly abundant in those of stem and inflorescence, which was consistent with a previous report<sup>10</sup> (Figure 2B). These results demonstrated the LMW RNA extracted by this method contained abundant small RNA and could be used for further applications, such as expression analysis.

In the routine nucleic acid extraction recipe, a high concentration sodium chloride (usually 1.4 M) is included for the purpose of dissolving the protein-nucleic acid complexes. Successfully extracting liver tissue with gradient concentrations of NaCl resulted in different recovery rates for DNP and RNP,<sup>9</sup> which suggested that DNA, HMW RNA and LMW RNA have different solubilities in certain concentrations of salt buffers. We tested several kinds of salts and found 0.4 M NaAC was the most effective for separating LMW RNA from DNA and HMW RNA. Using low concentrations of NaAC, we developed a fast and efficient method to isolate LMW RNA from *Arabidopsis* tissue. In this method, DNA and HMW RNA were precipitated during the first centrifuging and LMW RNA remained in the supernatant. Therefore, the next step was just to recover the LMW RNA from the supernatant. Combined with the use of a silica



**Figure 1.** Low-molecular-weight (LMW) RNA (3 g) samples from different *Arabidopsis* tissues were run on urea (8%) 15% polyacrylamide electrophoresis gels (20 volts/cm, 2 h), and stained with ethidium bromide (EB). Lane L, leaf; Lane R, root; Lane S, stem; Lane I, inflorescence; Lane M, 67-nt DNA molecular marker.



**Figure 2.** Northern blotting of *Arabidopsis* microRNA MIR319C. 30 microgram LMW RNA extracted from different *Arabidopsis* tissues using LSC buffer were run on 15% denaturing urea PAGE gel (20 volts/cm, 1 h). (A) Photograph of ethidium bromide stained PAGE gel. (B) Blotting result using the 5'-digoxigenin labeled complement probe of MIR319C. Lane M, 10 pg 20 and 24 nt digoxigenin labeled DNA marker; Lane L, leaf; Lane R, root; Lane S, stem; Lane I, inflorescence; LMW RNA, band of low-molecular-weight RNA; FF, band of xylene cyanol FF; BB, band of bromophenol blue.

spin column for small scale extraction and double precipitation for large scale, this method produced LMW RNA with good quality and low polysaccharide and protein contamination. The NaAC concentration in LSC buffer was the key factor influencing the final quality of LMW RNA. We tested NaAC concentration of 0.1 M to 0.6 M and found the best results could be obtained at 0.4 M. Higher concentration led to DNA contamination, while a lower concentration resulted in poor yield (*data not show*). This method can also be used for extracting LMW RNA from other plants (*Supplementary Figure 1*). For example, we have extracted LMW RNA from rubber tree using this method and constructed a microRNA library (*ZW An, unpublished data, 2010*).

In summary, we have developed a simple and fast extraction method for the isolation of LMW RNA from *Arabidopsis* tissues using LSC buffer. The method was quick and efficient, isolating LMW RNA from tissue in one step and

no extra procedure was needed for the elimination of DNA and HMW RNA. The total extraction process took no more than one hour for small scale extraction, and even only three to four hours for large scale extraction, while the yield and RNA quality were comparable with those published.<sup>8</sup> In addition, the LMW RNA isolated using this method was free of HMW RNA and abundant in small RNA. This method can be used for extracting LMW RNA for the purpose of small RNA cloning and detection, and library construction.

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