

A Novel Method To Polyadenylate Plant MicroRNAs Containing a 2'-O-Methyl Group on the 3'-Terminal Ribose

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Introduction

Small RNAs are important post-transcriptional regulators of plant development and other physiological processes. An essential step for small-RNA analysis by deep sequencing, cloning, or microarrays is tagging the 3' end of these molecules with a known sequence, which can then serve as a primer-binding site for cDNA synthesis. Polyadenylation using poly(A) polymerase is a rapid and efficient method to accomplish this goal. However, some small RNAs (e.g., siRNAs, piRNAs, and plant miRNAs) contain a 2'-O-methyl (Me) group at the 3'-terminal ribose, and this modification prevents the RNA from being an initiator for poly(A) polymerase.

RNA ligases, such as T4 RNA ligase 1 and 2, are less sensitive to this modification and are useful to append an oligonucleotide adaptor to the 3' end of RNAs. However, efficient ligation requires large excess of adaptor that needs to be removed from the ligation products, e.g., by gel electrophoresis.

We have developed a novel method to polyadenylate RNA molecules that contain the 2'-O-Me modification. Our method does not require the use of longer oligonucleotide adaptors. The products of ligation may be used directly for downstream analysis, such as cDNA synthesis, without purification, or they may be purified using simple procedures. We demonstrate the utility of this method to characterize modified RNAs present in mixtures, following methods to enrich for RNAs containing the 2'-O-Me group.

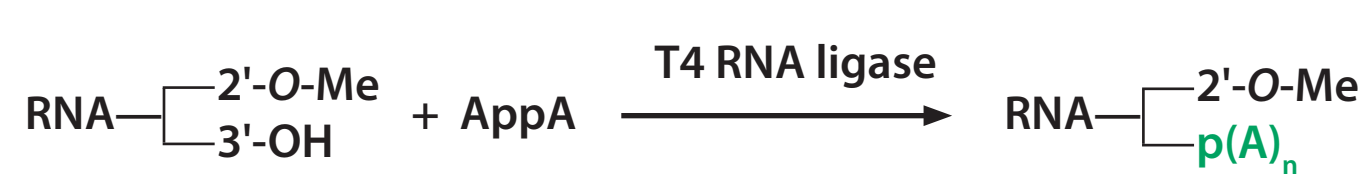
Methods and Results

Polyadenylation with T4 RNA ligase and diadenosyl pyrophosphate (AppA)

T4 RNA ligase catalyzes the reaction between adenosine pyrophosphate dinucleotides and RNA with a 3'-hydroxyl group (England, TE et al. [1977] *Proc. Natl. Acad. Sci. USA* 74:4839-42).



In the presence of diadenosyl pyrophosphate (AppA), T4 RNA ligase adds a poly(A) tail to 2'-O-methylated RNA, e.g. plant miRNA molecules.



AppA (Fig. 1) was synthesized according to Moffatt, JG and Khorana, HG (1961) *J. Am. Chem. Soc.* 83:649-658. AppA was purified by anion-exchange chromatography, and its purity was checked by thin-layer chromatography on Silica Gel 60 F254 plates.

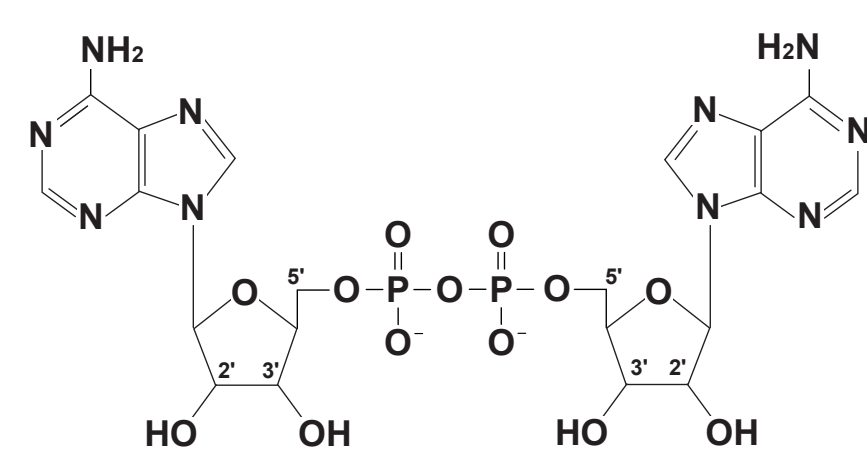


Figure 1. Structure of diadenosyl pyrophosphate (AppA).

Five picomoles of synthetic miR173 RNA, 2'-O-methylated at its 3'-terminal ribose (IDT), was incubated at room temperature with 2 µg of T4 RNA Ligase 2, 0.5 µl of APex™ Heat-Labile Alkaline Phosphatase (EPICENTRE), 1 mM AppA, in the presence of 20% DMSO, 10 U ScriptGuard™ RNase Inhibitor (EPICENTRE), 10 mM DTT, 25 mM Tris (pH 8.0), 1 mM MgCl₂, and 50 mM NaCl (Fig. 2A). Increasing amounts of miR173 (2'-O-Me) RNA was polyadenylated as described for Fig. 2A, for 6 hours (Fig. 2B). Five picomoles of *in vitro*-transcribed RNA 51-mer was incubated for 4 hours, as described for Fig. 2A (Fig. 2C). The products were analyzed by 16% acrylamide/8M urea PAGE.

RNA with or without a 2'-O-Me group on the 3' ribose was polyadenylated.

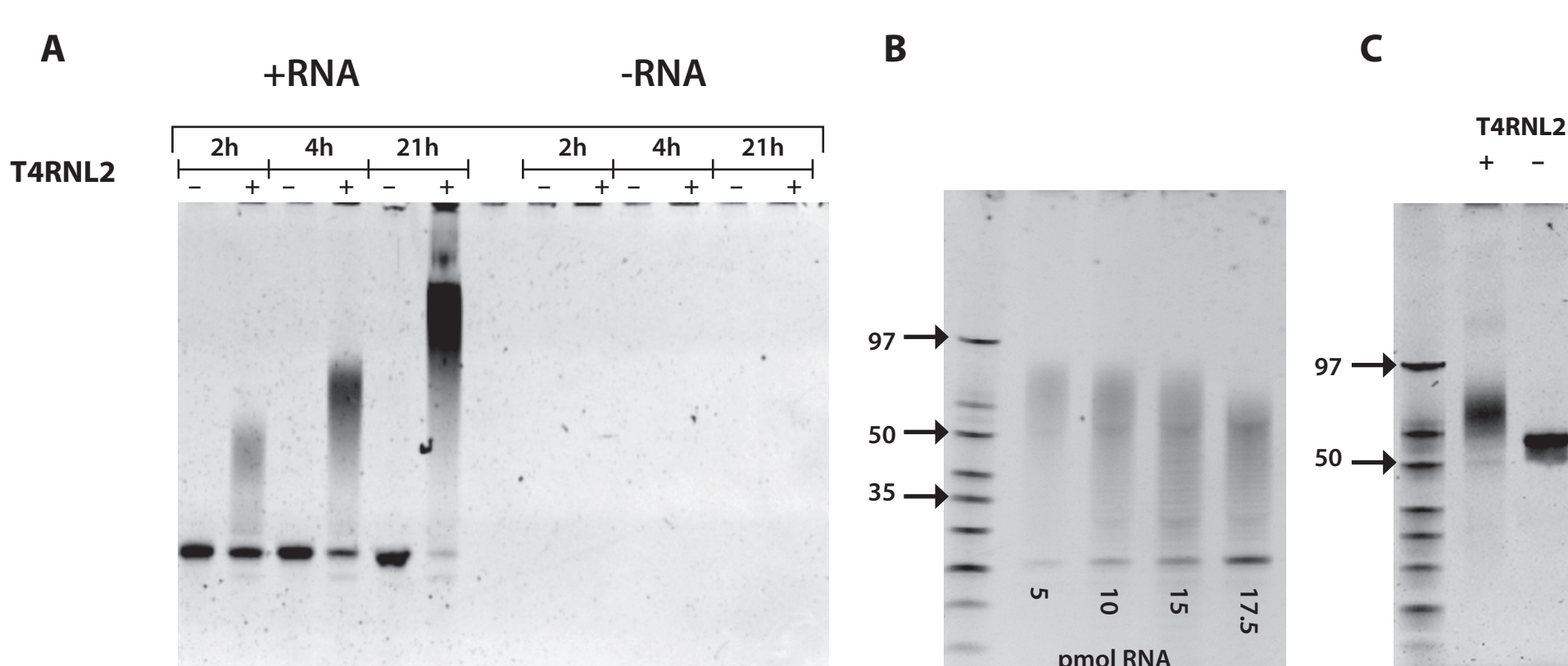


Figure 2. Polyadenylation of a synthetic miRNA. T4RN2, T4 RNA Ligase 2.

Detection of a 2'-O-Me miRNA spike in human RNA

Total HeLa RNA was spiked with synthetic miR173 (2'-O-Me) RNA at 4 fmol/µg total RNA. The low-molecular-weight (LMW) (<150 nt) RNA fraction was obtained by precipitation, and polyadenylated with AppA and T4 RNA ligase as described in Fig. 2A. The reaction mixture was preincubated for 30 minutes at room temperature before the addition of ligase in order to dephosphorylate the 5' ends of the RNA. The reaction was stopped with 1.5 mM EDTA, and incubated at 70° for 15 minutes to inactivate APex™ Alkaline Phosphatase. An aliquot of the tailed RNA was reverse-transcribed without further purification, using a tagged oligo(dT) primer, and the MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (EPICENTRE). Dilutions of the cDNA were analyzed by end-point (Fig. 3A) and real-time (Fig. 3B) PCR, with a tag-specific 3' primer and miRNA sequence-specific 5' primer, and FailSafe™ PCR reagents (EPICENTRE).

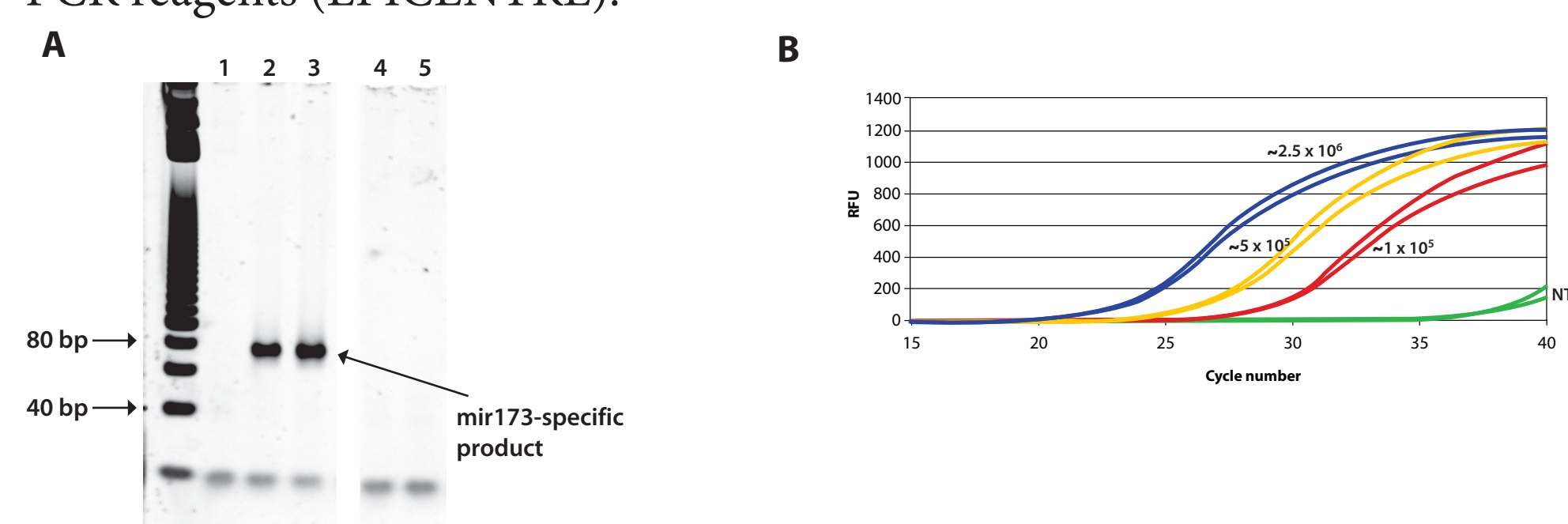


Figure 3. Detection of a 2'-O-Me RNA spike in human RNA. A) Lane 1, untailed RNA; lanes 2-3, poly(A)-tailed RNA; lane 4, poly(A)-tailed RNA, no RT; lane 5, no-template control. B) Real-time PCR plot of cDNA. Numbers represent the copies of spike-in RNA at the respective dilutions. NTC, no-template control.

cDNA synthesis from small RNA with a 2'-O-Me group at the 3'-terminal ribose

(Also see Poster #P042 by Vaidyanathan *et al.*)

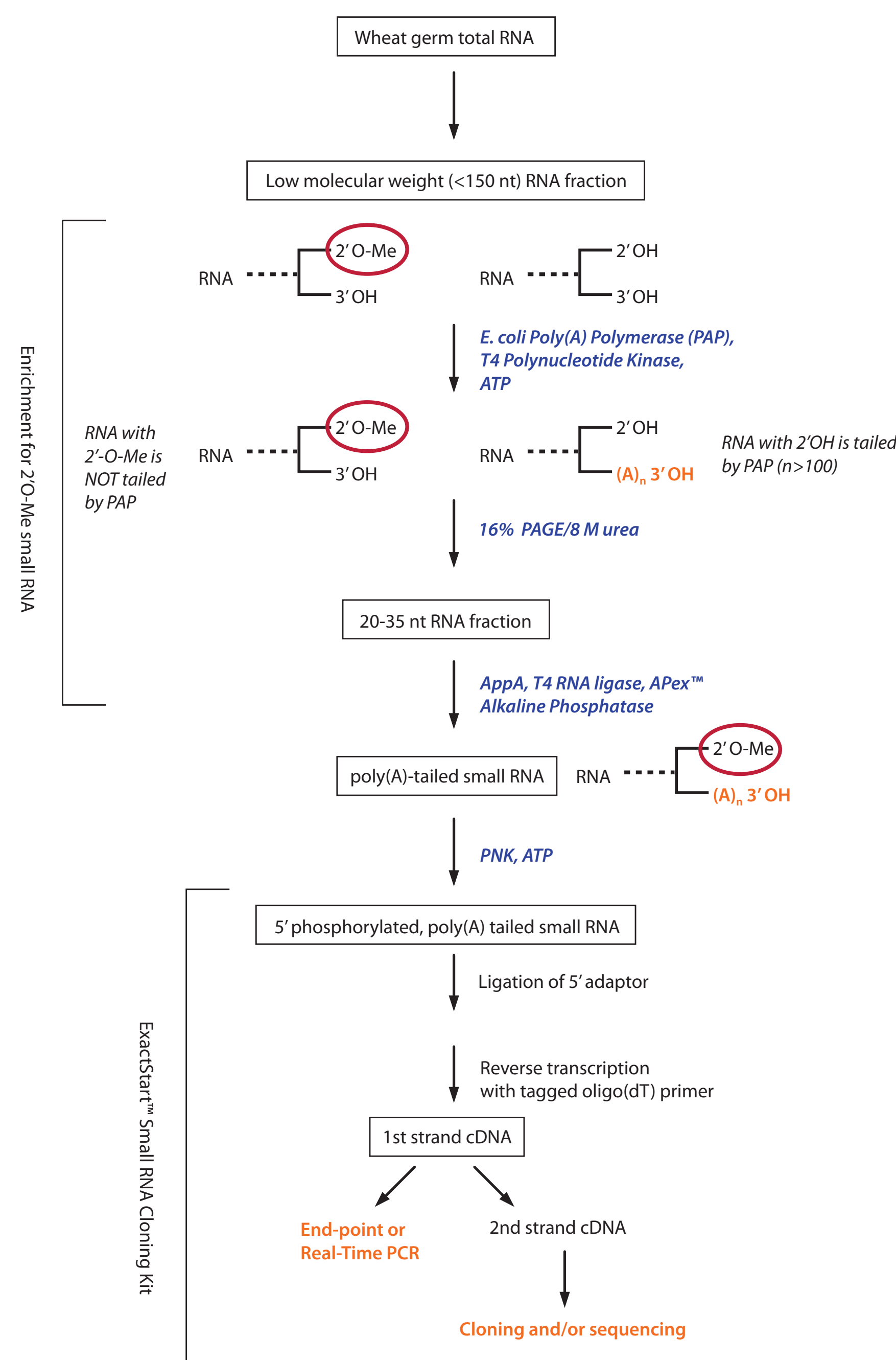


Figure 4. Schematic overview of the cDNA synthesis process.

Detection of endogenous plant miRNAs

Soybean leaf RNA

Soybean leaf total RNA was isolated with the MasterPure™ Plant RNA Purification Kit (EPICENTRE). The LMW (<150 nt) fraction of this RNA material was prepared by precipitation, using the Small RNA Enrichment Solution from the ExactSTART™ Small RNA Cloning Kit (EPICENTRE). For detection of known miRNAs from the LMW fraction, 420 ng of RNA was polyadenylated. For miRNA detection from total RNA, RNA was treated with sodium periodate to oxidize the terminal 2' and 3' OH groups, according to Igloi, GL and Kössel, H (1985), *Nucleic Acids Res.* 13:6885-6898. This treatment suppresses polyadenylation of RNA molecules with an unmethylated 2' OH group on the 3' end. The polyadenylation reaction contained ~1 µg of periodate-treated total RNA.

RNA was polyadenylated with AppA and T4 RNA ligase for 5 hours, as described for Fig. 2A. The reaction mixtures were preincubated for 30 minutes at room temperature before the addition of ligase. A portion (35%) of the polyadenylated RNA was reverse transcribed using a tagged oligo(dT) primer, and the MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (EPICENTRE). For miRNA detection by end-point PCR, cDNA derived from LMW RNA was diluted 500-fold, and cDNA derived from total RNA was diluted 50-fold. A 1-µl aliquot of diluted cDNA was used as PCR template. Specific primers were designed based on sequences in miRBase (www.mirbase.org). The expected size of the specific product is 62-70 bp (Fig. 5A).

Wheat germ RNA

For RNA isolation, wheat germ was homogenized in ice-cold buffer (50 mM Tris [pH 7.5], 1 mM EDTA, 5 mM MgCl₂, 0.1% β-mercaptoethanol, 5% glycerol). The cell debris was removed by centrifugation. The supernatant was digested for 1 hour at 37°C with 100 µg/ml proteinase K in the presence of 2% SDS, 10 mM EDTA, and 100 mM NaCl. LMW RNA (<150 nt) was prepared by isopropanol fractionation, and further purified by phenol-chloroform extraction. RNA of 20-35 nt length, enriched in molecules 2'-O-methylated on the 3' end was prepared, polyadenylated, and converted into cDNA as described for Fig. 4A. A 100-ng aliquot of gel-purified, 20-35 nt RNA was used. Identical miRNA-specific primers were used with both wheat germ and soybean leaf samples.

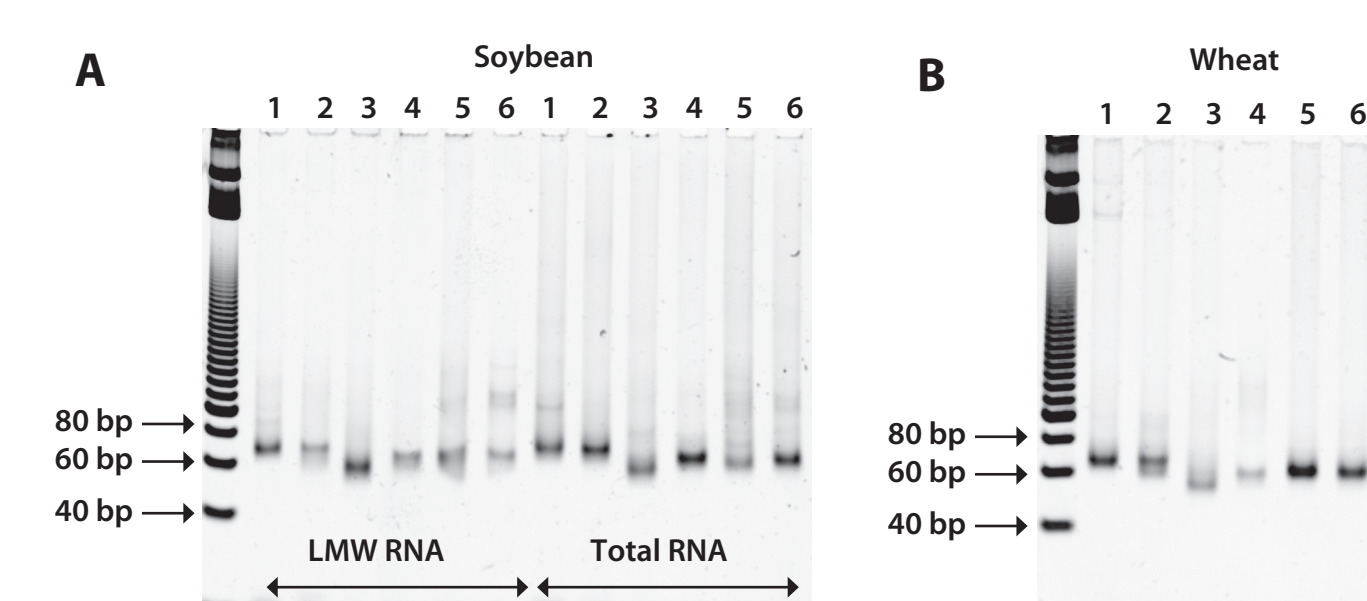


Figure 5. Detection of endogenous plant miRNAs. A) miRNAs from soybean leaf RNA. B) miRNAs from 2'-O-Me-RNA enriched, <35 nt fraction of wheat germ RNA. Lanes 1-6, miR156, miR159, miR164, miR167, miR169, and miR172, respectively.

The soybean leaf-derived cDNA samples were also used as templates in real-time PCR (Table 1). The similarity of C_t values suggests that miRNAs can be quantified directly from total RNA, after polyadenylation with AppA and T4 RNA ligase, and cDNA synthesis with a tagged oligo(dT) primer.

Table 1. Real-time PCR of endogenous plant miRNAs.

miRNA	C _t Soy LMW RNA	C _t Soy Total RNA
miR159	19.27	18.89
miR164	18.56	17.47
miR167	20.68	20.20
miR169	21.15	19.45
miR173	28.00	27.51

Sequencing of wheat germ small-RNA library

Wheat germ small (20-35 nt) RNA, enriched in molecules containing 3'-OH, 2'-O-Me termini, was used for cDNA library construction as shown in Figure 4. Insert sequences from ~50 clones, with inserts ≥15 bp, were aligned to wheat (*Triticum aestivum*, taxid: 4565) expressed sequence tags (ESTs) using the BLASTn 2.2.22 program (Altschul, SF et al. [1997], *Nucleic Acids Res.* 25: 3389-3402). Table 2 shows best matches with E values <1. When multiple matches of equal score and expect (E) value were found, one with a plus/minus match is shown.

Table 2. Analysis of small-RNA sequence matches to EST database.

Clone ID	Sequence	Length	Accession #	5' of Match	3' of Match	E Value
15	CATGTCAAATTCGGTCTACCCGGC	24	gb CA681553.1	150	127	2.00E-05
83	CGTCGGACGCGCCGGTACCCGGC	24	gb GH729168.1	114	91	2.00E-05
54/a	GCCCGCCGCGCCGGTACCCGGC	45	gb CA718236.1	34	11	6.00E-05
4	TTCTTAATCTGTTTCCCAATGCCG	23	dbj C1864125.1	599	619	9.00E-04
85	TACCTGGTGTATCCTCCCaG	20	gb FL645993.1	60	41	0.002
54/b	GCCCGCCGCGCCGGTACCCGGC	45	gb GH729121.1	240	220	0.003
6	TATGCTGAAGGATGATGACCAATC	24	dbj C1714466.1	156	133	0.004
77	CTTTCTTATTCCTCGTITTCaAG	25	gb CK161856.1	271	248	0.004
44	GCACGCTGTCCGGGACCCG	19	gb GH729189.1	597	579	0.006
11	ATTGTTGTCTCTTTGAT	20	dbj C1952920.1	62	44	0.008
31	AGCGGTGCGGCTGACGGCG	20	gb EV254283.1	479	462	0.03
31	AGCGGTGCGGCTGACGGCG	20	gb EV254283.1	479	462	0.03
95	GGAGTCTGACATGCTGCaG	20	gb GH723770.1	725	708	0.03
27	GTNGGCAACGATGGCGCTTAGGC	25	dbj C1584101.1	510	489	0.068
29	CATAGTAGGATGCTTGAACCG	22	gb CD890727.1	144	129	0.71
69	TTTTCTGACCTACTGATaCAA	23	dbj C1907739.1	702	687	0.83
20	TTCCCTGGATGCGCaCC	17	gb BE402975.1	930	916	0.94
43	CCGCCCCGACGTCG	15	gb GH729233.1	153	139	0.94
78	TACCTGGTGTATCCT	15	gb FL645993.1	60	46	0.94
36	TCTCTTCTCCCTCATTTGCTATC	24	gb CV774241.1	567	582	0.95
71	TAGCGCTTAATCAAAATCTCAC	24	dbj C1582704.1	21	2	0.95
75	CGCAAAAATTTTAAAGAGCCGGC	24	gb CV759267.1	737	722	0.95

Conclusions

- Plant miRNA molecules, which are refractory to poly(A) tailing by poly(A) polymerase due to a 2'-O-methyl group on the 3'-terminal ribose, can be polyadenylated using T4 RNA ligase and AppA.
- The polyadenylated RNA can be reverse-transcribed into cDNA using an oligo(dT) primer, without intervening purification.
- Plant miRNA, tailed using AppA and T4 RNA ligase, can be efficiently converted into cDNA for quantification by real-time PCR, sequencing, or cloning.

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