

Small-RNA Transcriptome Analysis: A Rapid and Simple Method for Generating cDNA from End-Tagged RNA

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Introduction

Small noncoding RNAs are found in a wide variety of organisms from bacteria to humans. The generation of small-RNA libraries (for applications such as sequencing analysis) is essential to the understanding of their diverse biological functions.¹ Current methods to analyze the small-RNA transcriptome of a cell rely on introducing defined sequences (tags) to the ends of RNA or cDNA. Tagging the ends of RNA has the advantage of precisely identifying the 5' end and is therefore desirable. The widely used ligation-based methods for tagging the ends of RNA suffer from limitations such as the following:

- Selection of only monophosphorylated RNA (p-RNA) and consequent exclusion of RNA molecules containing other 5'-end groups, such as cap (Gppp-RNA) or triphosphate (ppp-RNA).
- The requirement for intervening steps (e.g., gel purification) to remove excess adapters.
- Additional steps that introduce unwanted exposure to RNA.
- A lengthy protocol.

The ExactSTART™ Platform of tools for transcriptome discovery and analysis overcomes these limitations and provides the ability to tag RNA molecules based on the nature of the 5'-end group (p-RNA, ppp-RNA, Gppp-RNA, HO-RNA) without any intervening gel purification steps. Further, the amplified end-tagged ds cDNA is ready in less than a day (4-6 hours) and can be tailored to suit a number of applications.

The ExactSTART End-Tagged Double-Strand cDNA Synthesis Kit for Small RNA* includes the enzyme RNA 5' Polyphosphatase* that sequentially removes the γ and β phosphates from ppp-RNA to yield p-RNA, which serves as the donor molecule in the 5'-end tagging reaction. The resulting end-tagged RNA is reverse-transcribed and amplified to yield ds cDNA (Fig. 1), which can be used for a variety of applications. The cDNA can also be used to generate a small-RNA cloned library, with the ExactSTART Small RNA Cloning Kit.*

*Covered by issued and/or pending patents; see www.EpiBio.com/legal.

Methods and Results

Overview

A schematic overview of the process for generating end-tagged RNA and conversion to cDNA is shown (Fig. 1) with steps summarized below:

Small-RNA enrichment: Large RNAs, greater than ~200 bases (such as 18S and 28S rRNA, and mRNA) are selectively precipitated from the total RNA sample using the Small RNA Enrichment Solution. Small RNAs, such as miRNA, other small noncoding RNAs, tRNA, and small rRNA do not precipitate and remain in the soluble fraction.

5'-end modification (optional): The enzyme RNA 5' Polyphosphatase (see p. 21) sequentially removes the γ and β phosphates from 5'-triphosphorylated RNA to yield RNA with a 5'-monophosphorylated end, which is required for 5'-end tagging. This step is necessary only if you wish to include 5'-triphosphorylated RNA in the final population of ds cDNA.

3'-poly(A) tailing: A poly(A) tail is added to the 3' end of the RNAs, thus providing a priming site for cDNA synthesis. *Note:* Plant miRNAs will not be tailed (see p. 9).

5'-end tagging: Only 5'-monophosphorylated RNAs are substrates for 5' tagging by ligation of the Small RNA Acceptor Oligo (which contains a PCR priming site) using RNA Ligase.

cDNA synthesis: The tagged RNAs are reverse-transcribed into cDNA, using the cDNA Synthesis Primer that consists of an oligo(dT) sequence with a PCR priming site at its 5' end.

Second-strand cDNA synthesis and PCR: PCR is used to produce the second strand and amplify the resulting ds cDNA. PCR primers anneal to the priming sites at the 5' and 3' ends of the cDNAs. Only those cDNAs that contain *both* primer sequences will be converted to amplified ds cDNA. Other application-specific PCR primers can also be used, e.g., next-gen sequencing adapters.

The following additional steps are taken when using the ExactSTART Small RNA Cloning Kit:

Digestion with restriction enzymes: In addition to PCR priming sites, the 5'- and 3'-end tags contain *Not* I and *Asc* I restriction sites for directional cloning of the DNA into the pCDC1-K™ Cloning-Ready vector provided.

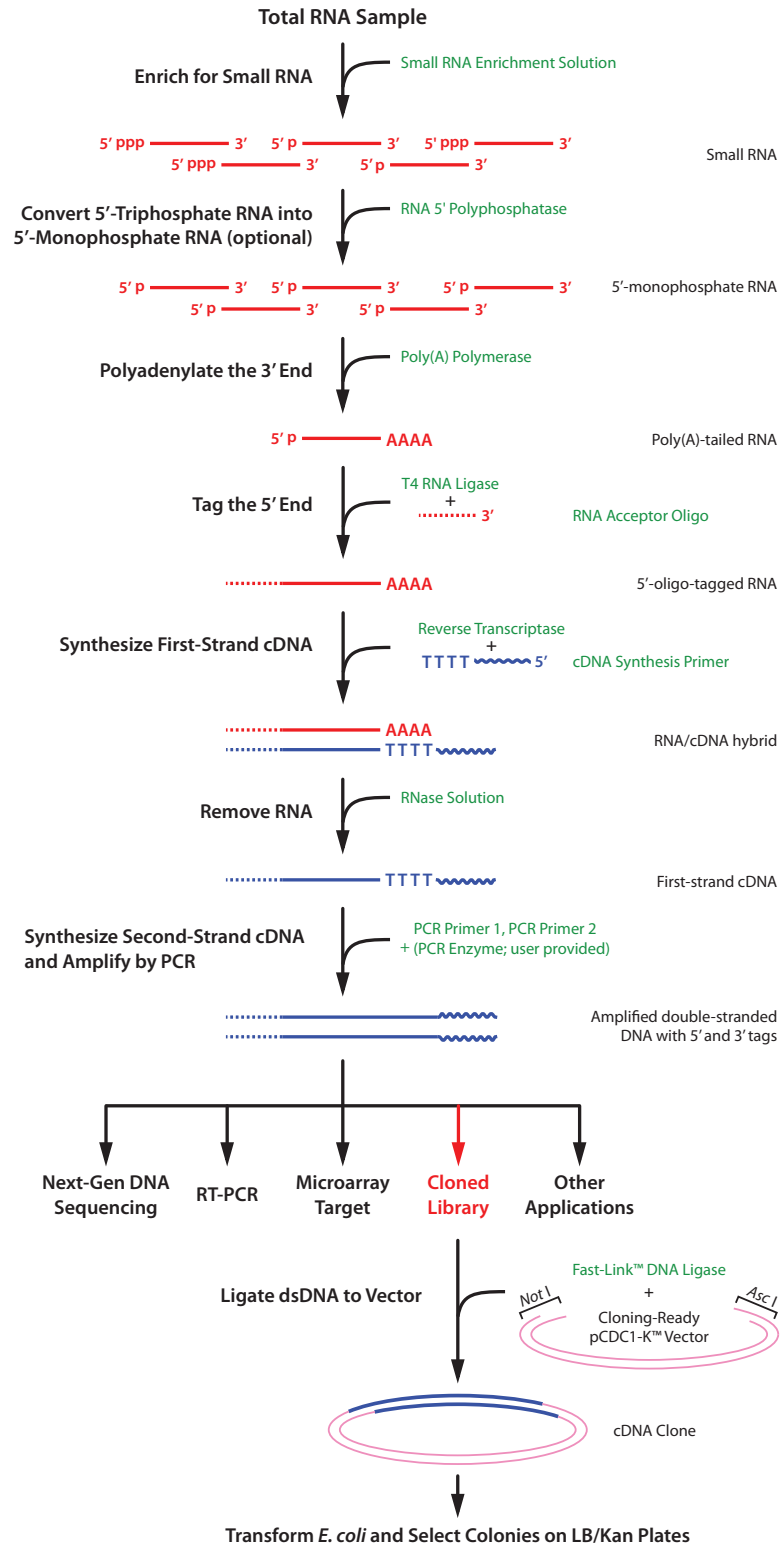


Figure 1. Schematic overview of the ExactSTART™ Kits for small-RNA analysis. Both small 5'-monophosphorylated RNAs, such as miRNAs, and small 5'-triphosphorylated RNAs are efficiently converted to 5'- and 3'-end tagged ds cDNA. The cDNA can be used to generate a cloned library with the ExactSTART Small RNA Cloning Kit.

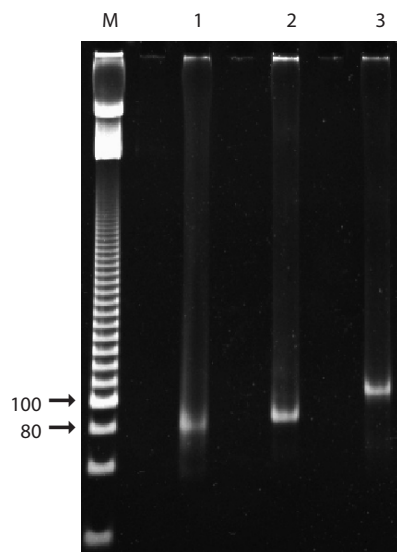


Figure 2. Double-strand cDNA generated from both small 5'-monophosphorylated RNAs and 5'-triphosphorylated RNAs. Using the ExactSTART™ procedure as outlined in Fig. 1, cDNA was produced from HeLa RNA (18- to 24-nucleotide transcripts; 5'-monophosphorylated) and from 5'-triphosphorylated synthetic transcripts (30 and 51 nucleotides, respectively). Products were separated on a 10% polyacrylamide gel. Lane M, 100-bp DNA ladder; lane 1, HeLa RNA; lane 2, 30-nucleotide transcript; lane 3, 51-nucleotide transcript. Note: The 5'- and 3'-end tagging process adds ~60 nucleotides to the final ds cDNA product.

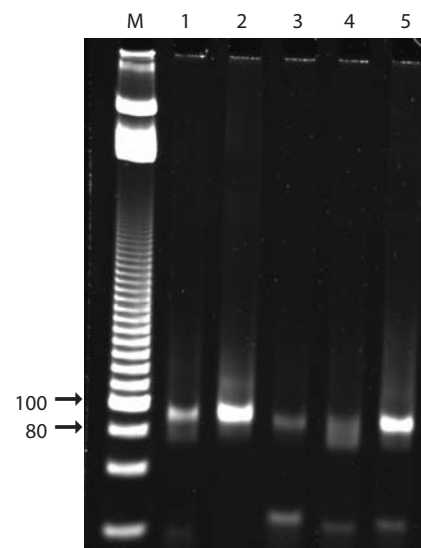


Figure 3. Detection of specific miRNAs in HeLa cells by PCR. Lane M, DNA marker; lanes 1-5, PCR products of cDNAs corresponding to miR16, miR21, miR26ab, miR29, and miR32, respectively.

Small RNAs with 5'-monophosphorylated ends or 5'-triphosphorylated ends can be converted to ds cDNA in less than 1 day.

Gel purification: The restriction-digested DNA is size-selected by gel electrophoresis and purification.

Vector ligation and cloning: The ds cDNA is ligated into the *Not* I/*Asc* I sites of the linearized and dephosphorylated pCDC1-K Cloning-Ready Vector provided. Competent *E. coli* (provided by the user) are transformed and clones are grown overnight.

Construction of small-RNA libraries

Small RNA from the indicated sources was tagged using the scheme shown in Fig. 1, and the amplified ds cDNA was analyzed on a polyacrylamide gel (Fig. 2). DNA of size ~85 bp corresponds to tagged HeLa miRNA (Fig. 2).

Detection of specific miRNAs in small-RNA libraries by PCR

A low-molecular-weight RNA fraction (<200 nt) obtained from 10 µg of total RNA isolated from HeLa, NIH3T3, and NRK cells was used for tagging and amplification. miR16, miR21, miR26ab, miR29, and miR32 were detected in the HeLa library using primers specific to the respective miRNAs (Fig. 3, lanes 1-5). The same miRNAs were also detected in NIH3T3 and NRK cells (data not shown).

Quantitative detection of human miRNAs

Real-time PCR was used to detect specific human miRNAs in HeLa cells. For this purpose, first-strand cDNA (ss cDNA) prior to amplification, and the amplified ds cDNA, were used as templates. The relative abundance of miR16, miR21, miR26a/b, miR29, and miR32 in the unamplified and amplified samples was compared by real-time PCR (Table 1).

The C_T values showed that the order of abundance of the five human miRNAs is the same in unamplified and amplified cDNA. This suggests that the relative levels of miRNAs are not affected by amplification.

Identification of miRNA in a small-RNA library from HeLa cells

Size-fractionated small RNAs (18-24 nt) isolated from HeLa cells were tagged, reverse-transcribed, amplified, and cloned into the *Not* I + *Asc* I sites of the cloning-ready pCDC1-K Vector to generate a small-RNA library. Colonies were randomly picked and the insert DNA in 96 clones was analyzed by sequencing. This led to the identification of 20 characterized human miRNAs (<http://microrna.sanger.ac.uk>) shown in Table 2.

Identification of human miRNA in a small-RNA library from FFPE human brain tissue

After removing paraffin with D-limonene, total RNA was isolated from formalin-fixed, paraffin-embedded (FFPE) human brain tissue using the MasterPure™ RNA Purification Kit (EPICENTRE). A library of small-RNA clones composed exclusively of small 5'-monophosphorylated RNAs, such as miRNAs, was constructed using the ExactSTART Small RNA Cloning Kit. Colonies were randomly picked and sequenced, and the miRNA clones identified included the following: hsa-let-7a, hsa-let-7b, hsa-let-7e, hsa-let-7i, hsa-mir-22, hsa-mir-26a, hsa-mir-29b-1, hsa-mir-92a, hsa-mir-125b, hsa-mir-195, hsa-mir-323, hsa-mir-342, and hsa-mir-520e.

Table 1. Relative abundance of human miRNAs in unamplified ss cDNA and amplified ds cDNA. As measured by C_T values, the relative abundance of the miRNAs is not altered by the kit reaction.

miRNA	C_T ss cDNA (Unamplified)	C_T ds cDNA (Amplified)
hsa-miR-16	17.9	14.3
hsa-miR-21	16.7	13.4
hsa-miR-26a/b	22.3	17.8
hsa-miR-29	20.3	16.1
hsa-miR-32	18.8	15.8

Table 2. Identification of characterized human miRNAs in a HeLa cDNA library prepared using the ExactSTART™ Mammalian Small RNA Cloning Kit. Ninety-six randomly selected clones from the library were analyzed by sequencing.

miRNA	Hits	miRNA	Hits
hsa-let-7a	1	hsa-miR-27b	1
hsa-let-7i	1	hsa-miR-92a	7
hsa-miR-15b	1	hsa-miR-96	1
hsa-miR-18a	1	hsa-miR-99a	1
hsa-miR-19b	2	hsa-miR-125a-5p	1
hsa-miR-20a	2	hsa-miR-181a	1
hsa-miR-21	7	hsa-miR-196a	1
hsa-miR-23a	1	hsa-miR-221	1
hsa-miR-24	2	hsa-miR-365	1
hsa-miR-26a	1	hsa-miR-484	1

Distribution of small-RNA species in libraries prepared from plant tissue

Small-RNA libraries were prepared using RNA isolated from leaves of corn, rice, and wheat plants, and the libraries were analyzed by DNA sequencing. The distribution of small RNAs in these libraries is shown in Fig. 4. Note that plant miRNAs have a 2'-O-methyl group at the 3' end of the RNA, which greatly reduces the efficiency of tailing by Poly(A) Polymerase. As a result, miRNAs are underrepresented in the plant small-RNA libraries shown.

Conclusions

The ExactSTART End-Tagged Double-Strand cDNA Synthesis Kit for Small RNA uses a streamlined protocol to generate ds cDNA from small RNA. Small RNAs with 5'-monophosphorylated ends or 5'-triphosphorylated ends

can be converted to ds cDNA in less than 1 day. The ds cDNA can be used for a variety of applications, including next-gen sequencing, and preparation of a cloned library using the ExactSTART Mammalian Small RNA Cloning Kit.

References

1. Affymetrix/CSHL ENCODE Transcriptome Project, *Nature*, doi:10.1038/nature07759.

Cat. #	Quantity
ExactSTART™ End-Tagged Double-Strand cDNA Synthesis Kit for Small RNA ES81010	10 Reactions
ExactSTART™ Mammalian Small RNA Cloning Kit ES81005	5 Reactions

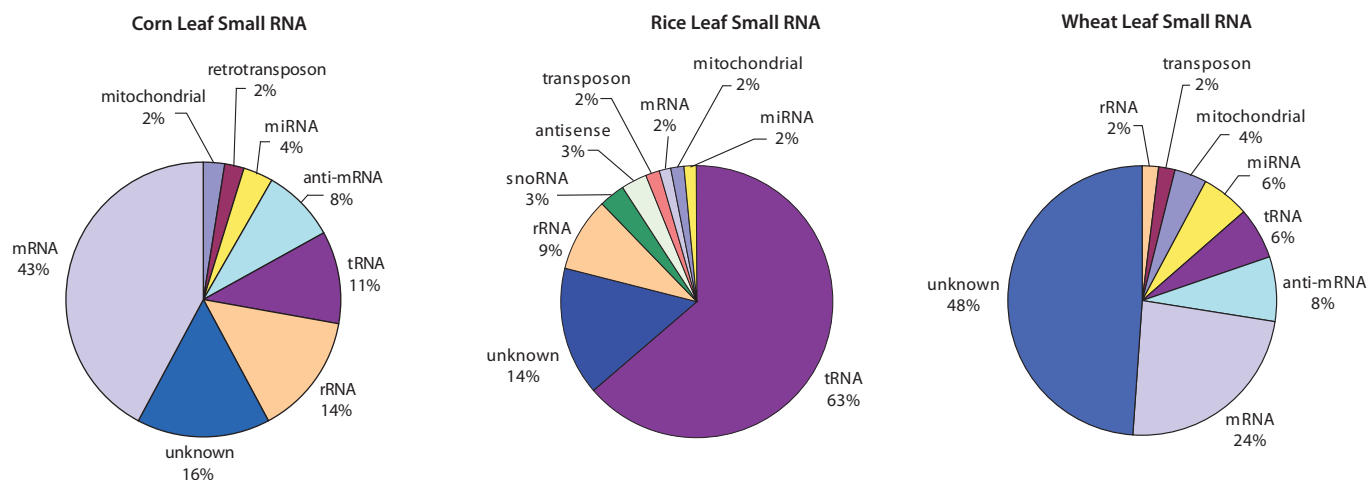


Figure 4. Relative abundance of various small-RNA species in plant-leaf tissue.