# Intramolecular ligation method (iLIME) for pre-miRNA quantification and sequencing

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## ABSTRACT

**Hairpin-containing pre-miRNAs, produced from pri-miRNAs, are precursors of miRNAs (microRNAs) that play essential roles in gene expression and various human diseases. Current qPCR-based methods used to quantify pre-miRNAs are not effective to discriminate between pre-miRNAs and their parental pri-miRNAs. Here, we developed the intramolecular ligation method (iLIME) to quantify and sequence pre-miRNAs specifically. This method utilizes T4 RNA ligase 1 to convert pre-miRNAs into circularized RNAs, allowing us to design PCR primers to quantify pre-miRNAs, but not their parental pri-miRNAs. In addition, the iLIME also enables us to sequence the ends of pre-miRNAs using next-generation sequencing. Therefore, this method offers a simple and effective way to quantify and sequence pre-miRNAs, so it will be highly beneficial for investigating pre-miRNAs when addressing research questions and medical applications.**

**Keywords: microRNA; pre-miRNA; pre-miRNA quantification; pre-miRNA sequencing; pri-miRNA**

## INTRODUCTION

MicroRNAs (miRNAs) play important roles in gene regulation, via triggering degradation or translational inhibition of their target messenger RNAs (mRNAs) (Friedman et al. 2009; Ameres and Zamore 2013; Jonas and Izaurralde 2015). In humans, more than 2500 miRNAs have been identified (Griffiths-Jones et al. 2006; Kozomara et al. 2019). They are critical for nearly all cellular processes and many of them are related to numerous human diseases (Ha and Kim 2014; Rupaimoole and Slack 2017; Bartel 2018; Gebert and MacRae 2019). For example, abnormal expression of some miRNAs, including the let-7 family, as well as miR-4521, miR-155, and miR-142, is related to various cancers (Rupaimoole and Slack 2017; Liu et al. 2021; Winkle et al. 2021). Each miRNA is 21–22 nt long and is a part of a primary miRNA (pri-miRNA), which is synthesized by RNA polymerase II in the nucleus. The Microprocessor cleaves pri-miRNAs and in this way produces precursor miRNAs (pre-miRNAs) (Fig. 1A). Pre-miRNAs are stem–loop RNA structures that contain 1–3 nt overhang at their 3′-ends. They are exported from the nucleus to the cytoplasm by Exportin-5 and then are cleaved by DICER to generate miRNA duplexes of 21–22 nt (Ha and Kim 2014; Bartel 2018). Eventually, Argonaute (AGO) selects one strand of each miRNA duplex to form an AGO-miRNA complex. This forms the core of the miRNA-induced silencing complex (RISC), where miRNAs base pair with their target mRNAs. The RISC then executes degradation or translational suppression of these target mRNAs (Iwasaki and Tomari 2009; Jonas and Izaurralde 2015; Bartel 2018; Gebert and MacRae 2019). As sequences and relative levels of both pri-miRNAs and pre-miRNAs are essential for functions of miRNAs, abnormal expression levels of pri-miRNAs and pre-miRNAs are associated with various cellular defects and human diseases. To this end, multiple miRNAs are used as biomarkers for disease diagnosis (Condrat et al. 2020).

Since expression and sequence of pri-miRNAs, pre-miRNAs, and miRNAs are critical for numerous cellular functions and human diseases, there is a high demand for them to be quantified and sequenced (Ha and Kim 2014; Rupaimoole and Slack 2017; Bartel 2018; Forero et al. 2019; Gebert and MacRae 2019; Nguyen et al. 2019; Liu et al. 2021; Winkle et al. 2021). In the last 20-plus years, quantitative real-time polymerase chain reaction (qPCR) methods have been widely used to quantify pri-miRNAs and miRNAs (Chen et al. 2005; Forero et al. 2019). Pri-miRNAs are long RNA molecules consisting of anywhere between a few hundred to several thousand nucleotides, and traditionally the classical qPCR method has been used to quantify pri-miRNAs as well as other long RNAs,

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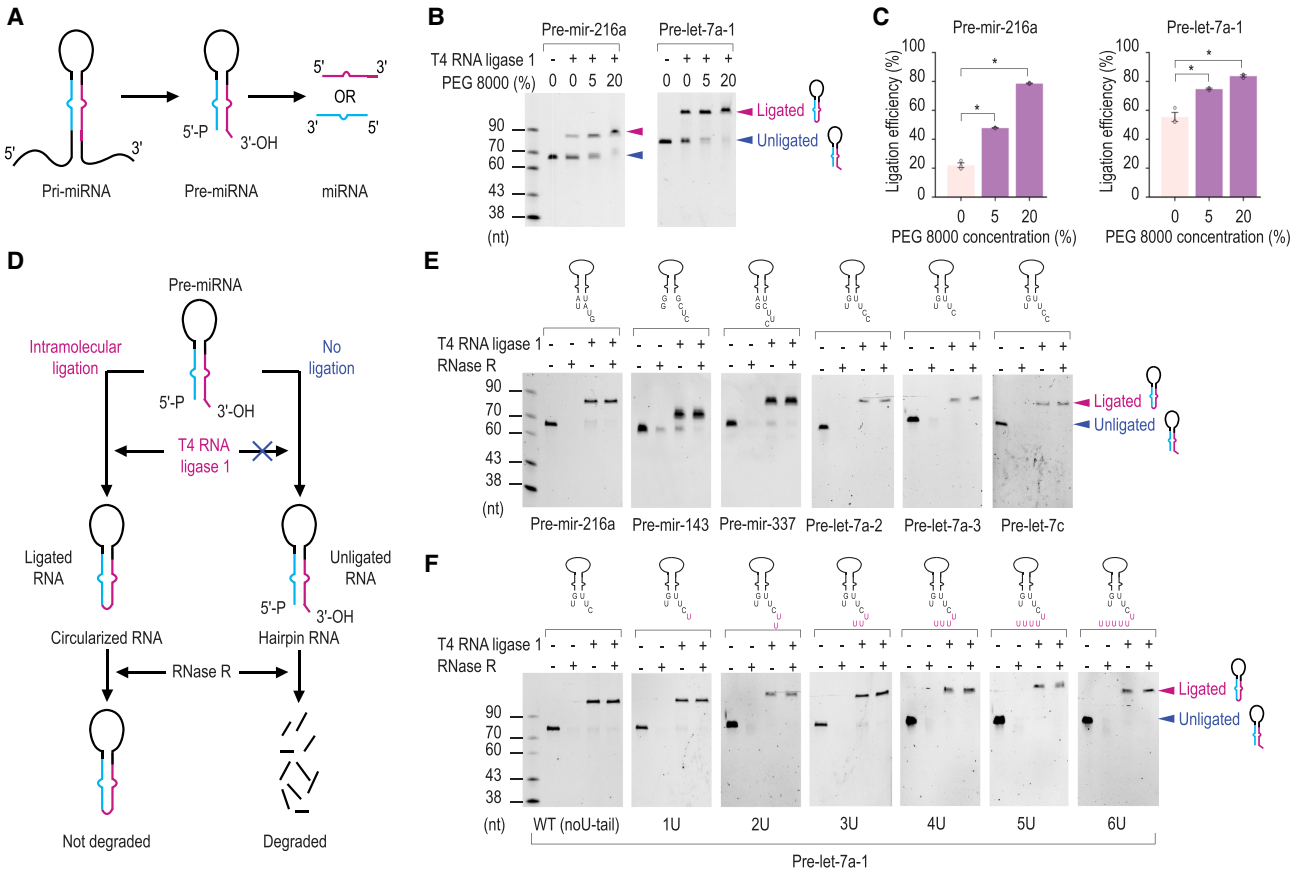


**FIGURE 1.** Circularization of pre-miRNAs by T4 RNA ligase 1. (*A*) Diagram to illustrate microRNA biogenesis. Each pre-miRNA contains a monophosphate at its 5′-end (5′-P) and a hydroxyl at its 3′-end (3′-OH). (*B*) Circularization of the pre-miRNA by T4 RNA ligase 1. Each pre-miRNA (1 pmol) was incubated with 16 units of T4 RNA ligase 1 at 25°C for 2 h in ligation buffer (NEB, M0204L) alone or supplemented with 5% or 20% polyethylene glycol 8000 (PEG 8000). The ligation reaction mixtures were then analyzed via 12% urea-PAGE. The ligated products and original substrates are indicated by red and blue arrowheads, respectively. (*C*) The ligation efficiency of the pre-miRNAs was calculated from three repeated experiments as the percentage of the original substrate that was converted into ligated product. Two-tailed two-sample assuming unequal variances *t*-tests for statistical analysis were used to calculate the *P*-value. (*) *P*-value <0.05. (*D*) The diagram shows ligated or unligated pre-miRNAs being treated with RNase R. The pre-miRNAs were ligated by T4 RNA ligase 1 as described in *B*. The ligated RNAs were purified by isopropanol and were subsequently treated with five units of RNase R at 37°C for 1 h. (*E*,*F*) The T4 RNA ligase 1-circularized pre-miRNAs were resistant to RNase R. One pmol of each pre-miRNA was used in these RNase R incubation experiments. Primer sequences used for RNA preparation are shown in Supplemental Table S1.

such as mRNAs or long noncoding RNAs. In contrast, miRNAs are short RNAs (just 21–22 nt in length), and so the classical qPCR method does not work. Several interesting methods have been developed to quantify miRNAs using qPCR (Forero et al. 2019). For example, stem–loop reverse transcription (RT) primers have been used to extend the length of the cDNA synthesized from miRNAs, or poly (A) has been added to elongate miRNAs (Chen et al. 2005; Shi and Chiang 2005). These qPCR methods developed for miRNAs can distinguish mature miRNAs from their parental pre-miRNAs and pri-miRNAs (Chen et al. 2005; Shi and Chiang 2005; Kumar et al. 2011). In addition, the qPCR method developed for pre-miRNAs can also distinguish them from miRNAs (Schmittgen et al. 2008). However, it is also essential for developing a qPCR method that can discriminate pre-miRNAs from pri-miRNAs. Unlike miRNAs and pri-miRNAs, development of the qPCR method for pre-miRNAs faces several technical difficulties. Only 1–3 nt at the 3′-end of pre-miRNAs are not base-paired, and so they are not long enough to make a base pair with the RT primers in the same way that stem–loop RT primers are used for miRNAs (Chen et al. 2005). In addition, each pre-miRNA is a part of its parental pri-miRNA, thus, the classical qPCR technique (Schmittgen et al. 2008) quantifies both the pre-miRNA and its parental pri-miRNA simultaneously. In fact, qPCR kits for pre-miRNAs are commercially available; however, on the company website, it is stated that the kits cannot discriminate between pre-miRNAs and pri-miRNAs.

Since fidelity of pri-miRNAs, pre-miRNAs, and miRNAs is crucial for their functions, another approach for studying these RNA sequences is to perform sequencing. A

common RNA-sequencing method involves converting RNA into DNA, and this is then sequenced by Sanger sequencing or next-generation sequencing (NGS) technology. Commonly, both 5′- and 3′-ends of RNAs are ligated to known sequences, called the 5′- and 3′-adapters, respectively. These adapters are used later in the reverse transcription and PCR steps. As a result, full-length RNAs (from the 5′-end to 3′-end) can be converted into DNA and then sequenced. This approach allows both pri-miRNAs and miRNAs to be sequenced, and various sequencing kits have been developed for these two RNA types. Examples include the TruSeq Small RNA Library Preparation Kit (Lau and Lee 2012), the NEBNext Small RNA Library Prep Set (NEB, E7330L); and the SMARTer smRNA Kit (Takara, 635031). However, pre-miRNA sequencing is complex since ligating its 5′-end to a 5′-adapter is inefficient because the 5′-end nt of pre-miRNA is often base-paired (Burke et al. 2014; Liu et al. 2014; Lama et al. 2019).

Many publications demonstrated that hairpin-containing RNA molecules, such as tRNAs, containing two 5′-ends and 3′-ends at proximity, could be ligated (Sherlin et al. 2001; Lang and Micura 2008; Stark and Rader 2014; Yoshinari et al. 2017). In addition, the synthesized pri-mir-31 (229 nt) containing a hairpin and two flanking single-stranded RNA regions (~74 nt each side) was also demonstrated to be circularized by T4 RNA ligase 1 (Zeng and Cullen 2005). Essentially, one endogenous human pre-let-7a (Basyuk et al. 2003) and plant precursors of osa-miR827a and osa-miR1874 could also be circularized by T4 RNA ligase 1 (Lacombe et al. 2008). In this study, we investigated generalization of the intramolecular ligation for pre-miRNAs, which might be the key to tackling the pre-miRNA quantification issue. We demonstrated that T4 RNA ligase 1 could efficiently ligate the 5′- and 3′-ends of various synthesized pre-miRNAs and their elongated variants in vitro, thus generating circularized RNAs. This offers us a way to exclusively quantify synthesized and endogenous pre-miRNAs in the circular form using PCR (iLIME-qPCR). In addition, such an intramolecular ligation of the pre-miRNA approach also enabled us to sequence 5′-end and 3′-end of pre-miRNAs (iLIME-seq). Thus, the iLIME-qPCR and iLIME-seq are useful for quantifying and sequencing pre-miRNAs.

## RESULTS

### Pre-miRNAs are ligated into circularized RNAs by T4 RNA ligase 1

T4 RNA ligase 1 is an enzyme that ligates a 5′-phosphoryl (5′-P)-terminated nucleic acid donor to a 3′-hydroxyl (3′-OH)-terminated nucleic acid acceptor, forming a 3′–5′ phosphodiester bond. We know pre-miRNA molecules contain 5′-P and 3′-OH groups at the two ends, and these groups are brought close to each other by their natural hairpin structure (Fig. 1A; Supplemental Fig. S1A) folded using RNAfold (Lorenz et al. 2011). Therefore, we tested if T4 RNA ligase 1 might catalyze an intramolecular ligation between these pre-miRNA terminal nucleotides. Interestingly, we found that the enzyme could connect these terminal nucleotides of pre-mir-216a and pre-let-7a-1, to form a "circularized" or "ligated" hairpin RNA structure. This circularized RNA ran slower in a urea polyacrylamide gel electrophoresis (urea-PAGE) than the "unligated" RNA (Fig. 1B, primer sequences used for RNA preparation are shown in Supplemental Table S1). Polyethylene glycol (PEG) is a well-known enhancer of T4 RNA ligase (Harrison and Zimmerman 1984), here we also showed that the addition of 20% PEG 8000 significantly increased the efficiency of the ligation reaction (Fig. 1B,C).

To confirm that the slower moving (upper) bands in the urea-PAGE were the intramolecularly ligated pre-miRNAs, we treated unligated and ligated pre-miRNAs with RNase R, an RNA exonuclease, which degrades RNAs at the 3′-OH end (Fig. 1D). We showed that RNase R removed all the unligated pre-miRNAs tested. In contrast, the ligated pre-miRNAs (upper bands in the gel) were unaffected by the addition of RNase R (Fig. 1E). These results of RNase R-treated experiments confirmed that the 3′-OH end of T4 RNA ligase 1-ligated pre-miRNAs was protected due to being ligated to the 5′-P end.

### Elongated pre-miRNAs are ligated into circularized RNAs by T4 RNA ligase 1

Many pre-miRNAs, including pre-let-7a-1, are known to be monouridylated or polyuridylated by TUTases (Liu et al. 2014; Kim et al. 2015; Treiber et al. 2019; Yu and Kim 2020). Therefore, we tested the capacity of T4 RNA ligase 1 to ligate uridylated pre-miRNAs. We synthesized pre-let-7a-1 containing an additional 1 to 6 uridines at the 3′-OH end (Fig. 1F; Supplemental Fig. S1A) and found that T4 RNA ligase 1 efficiently intramolecularly ligated all of these uridylated pre-miRNAs (Fig. 1F). In addition, T4 RNA ligase 1 also achieved similar ligation levels for 16 pre-let-7a-1 variants containing different combinations of 5′- and 3′-ends (Supplemental Fig. S1B). Taken together, we demonstrated that T4 RNA ligase 1 could efficiently ligate two ends of various pre-miRNAs and their derivatives, including the long-tailed ones.

### iLIME-qPCR for quantifying pre-miRNAs

We developed a qPCR method (iLIME-qPCR) to quantify pre-miRNAs precisely. The circularization of pre-miRNAs by T4 RNA ligase 1 allowed us to design a pair of primers that were oppositely directed to only amplify the circularized pre-miRNAs. Because of this opposite directionality, these primers should not amplify genomic DNA

or cDNA resulting from either unligated pre-miRNA or parental pri-miRNA (Fig. 2A). First, we performed normal PCR (without SYBR Green I) and found that the primers shown in Figure 2A could successfully amplify many circularized pre-miRNAs (including pre-mir-216a, pre-let-7a-1, pre-let-7a-2, pre-let-7a-3, and pre-let-7c) and generated PCR products with the expected length (Fig. 2B). In contrast, similar PCR reactions failed to amplify unligated pre-miRNAs or parental pri-miRNAs (Fig. 2B). These results indicate that the primers specifically amplify ligated pre-miRNAs.

We then performed iLIME-qPCR using SYBR Green I and 8 different primer sets (Supplemental Fig. S2A) and found that the primer set 7 and 8 showed similar $C_t$ values, which were lower than those of the other primer sets (Supplemental Fig. S2B). Besides, the PCR products resulting from iLIME-qPCR using the primer set 7 seemed more specific than primer set 8 (Supplemental Fig. S2C), thus we conducted subsequent iLIME-qPCR experiments using the primer set 7. Next, we used the set of primers designed for each of pre-mir-216a, pre-let-7a-1, pre-let-7a-2, pre-let-7a-3, and pre-let-7c, to amplify these five pre-miRNAs. We found that the primer sets did not amplify untargeted pre-miRNAs (Fig. 2C). These results indicate that iLIME-qPCR can quantify pre-miRNAs specifically. We then demonstrated that iLIME-qPCR could be used to quantify synthesized pre-miRNAs at a range of 0.0001–10 fmol (Fig. 2D). We then mixed the synthesized pre-miRNAs with the total RNA isolated from DROSHA-KO cells (which do not produce pre-miRNAs) and demonstrated that iLIME-qPCR could still accurately quantify pre-miRNAs when they were "contaminated" with other RNAs, as it did for the purified synthesized pre-miRNAs (Fig. 2E).

We then conducted iLIME-qPCR for the total RNAs isolated from parental HCT116, DROSHA-KO, and DICER-KO cells. In this experiment, we added an artificial pre-miRNA (which could be circularized and quantified by iLIME-qPCR) to the total RNAs and used it as a spike-in RNA (Supplemental Fig. S2D–F). Expectedly, we found that iLIME-qPCR detected significantly up-regulated levels of pre-miRNAs in the DICER-KO cells and extensively

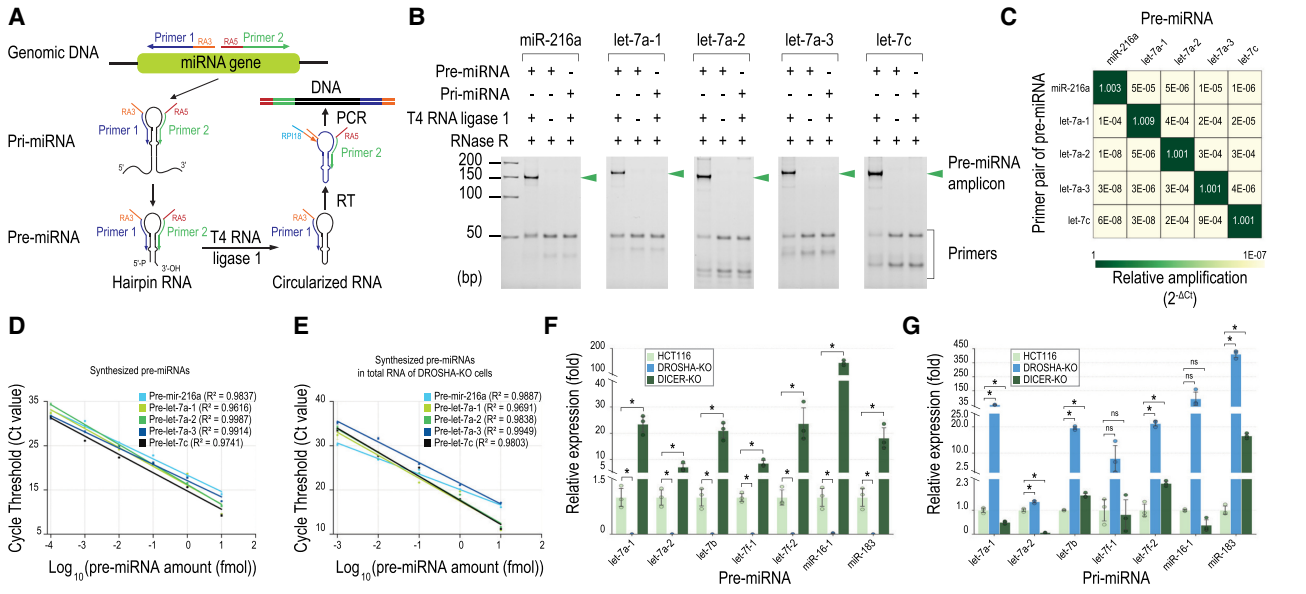


**FIGURE 2.** Quantification of pre-miRNAs by iLIME-qPCR. (*A*) Schematic diagram to illustrate the iLIME-qPCR. Pre-miRNA is first circularized by T4 RNA ligase 1. The circularized pre-miRNA is then reverse-transcribed to generate cDNA spanning the 5′-end and 3′-end of the pre-miRNA. The cDNA is then amplified with a pair of primers using PCR. (*B*) iLIME-PCR can amplify the circularized pre-miRNAs, but not the original hairpin pre-miRNAs or parental pri-miRNAs. iLIME-PCR was conducted for unligated pre-miRNAs, ligated pre-miRNAs, and their parental pri-miRNAs. (*C*) iLIME-qPCR specifically amplifies pre-miRNAs. This method was carried out similar to iLIME-PCR in Figure 2B except that SYBR Green I was added to the PCR reaction, the primer set 7 (Supplemental Fig. S2A) was used, and the PCR products were real-time quantified using the Roche LightCycler 480 real-time PCR system. The iLIME-qPCR primers designed for one pre-miRNA were also tested on four other pre-miRNAs. 10 fmol of each pre-miRNA were used in each qPCR reaction. The numbers shown on the heatmap were the average relative amplification values of three iLIME-qPCR repeats. The relative amplification was estimated as $2^{-\Delta Ct}$, such that the ΔCt was the difference between the Ct values obtained from the iLIME-qPCR using the primer set for one pre-miRNA and any of the other pre-miRNAs. (*D*) iLIME-qPCR can also be conducted on synthesized pre-miRNAs. Different amounts (i.e., from 0.0001 fmol to 10 fmol) of pre-miRNAs were tested. (*E*) iLIME-qPCR can also quantify synthesized pre-miRNAs added to the total RNAs isolated from DROSHA-KO cells. Different amounts (i.e., from 0.001 fmol to 10 fmol) of pre-miRNAs were tested. (*F*) iLIME-qPCR can also be used to estimate different expression levels of endogenous pre-miRNAs in HCT116, DROSHA-KO, and DICER-KO cells. Two-tailed two-sample assuming unequal variances *t*-tests for statistical analysis were used to calculate the *P*-value. (ns) Not significant, (*) *P*-value <0.05. (*G*) qPCR was used to estimate the expression levels of the endogenous parental pri-miRNAs of the pre-miRNAs shown in *F*. Two-tailed two-sample assuming unequal variances *t*-tests for statistical analysis were used to calculate the *P*-value. (ns) Not significant, (*) *P*-value <0.05. Primer sequences used for pre-miRNA and pri-miRNA quantification are shown in Supplemental Tables S2 and S3, respectively.

down-regulated levels of pre-miRNAs in the DROSHA-KO cells when compared with those in the parental HCT116 cells (Fig. 2F). These results indicate that iLIME-qPCR can measure endogenous pre-miRNAs. In parallel, we also conducted qPCR for pri-miRNAs and found that the levels of pri-miRNAs in the DROSHA-KO cells were expectedly disproportionate to those of the pre-miRNAs isolated from the HCT116 cells (Fig. 2G), confirming the defective pri-miRNA cleavage by the knockout of DROSHA. The expression levels of a few pri-miRNAs, including pri-let-7f-1 and pri-mir-16-1, were expectedly not different between the DICER-KO and parental HCT116 cells (Fig. 2G). However, some other pri-miRNAs, such as pri-let-7a-1, pri-let-7a-2, pri-let-7b, pri-let-7f-2, and pri-mir-183, showed different expressions between the DICER-KO and parental HCT116 cells, suggesting that those pri-miRNAs might be under regulation by miRNAs (Fig. 2G).

## Use of iLIME-seq to sequence pre-miRNAs

The Microprocessor cleaves pri-miRNAs and thus generates pre-miRNAs. Much of the time, this enzyme cleaves pri-miRNAs at multiple sites and produces several pre-miRNA isoforms containing different 5′- and 3′-ends (Ha and Kim 2014; Kim et al. 2017, 2021; Bartel 2018; Li et al. 2020). In addition, the 3′-end of pre-miRNAs can be modified by multiple enzymes that add or remove several nucleotides. These modifications might serve as a signal for the degradation or stabilization of pre-miRNAs, or they might increase pre-miRNA cleavage by DICER (Ha and Kim 2014; Bartel 2018; Treiber et al. 2019). Therefore, the 5′- and 3′-end identities of pre-miRNAs contain important information to help us understand how pre-miRNAs are generated and processed. Here, we developed a simple and convenient method, called iLIME-seq, for sequencing the 5′- and 3′-ends of targeted pre-miRNAs.

In brief, the circularized pre-miRNAs generated by T4 RNA ligase 1 were converted into DNA libraries using one RT step and two PCR steps, as shown in Figure 3A. The resulting DNA libraries were then sequenced by NGS. First, we used iLIME-seq to sequence the synthesized pre-miRNAs. The sequencing results showed that this method revealed the 5′- and 3′-ends of many tested pre-miRNAs accurately such that we obtained 85.4%–97.6% reads mapping the expected sequences of the synthesized pre-miRNAs (Fig. 3B,C; Supplemental Fig. S3A). A few percentages of the reads contained sequences that were several nt shorter or longer than at the 3′-ends of pre-miRNAs (Fig. 3B,C; Supplemental Fig. S3A). These pre-miRNA variants might be produced in the in vitro transcription (IVT) reactions by T7 RNA polymerases (Gholamalipour et al. 2018).

We then used iLIME-seq to sequence pre-let-7a-1 containing different uridine tails (U-tails) and again obtained the expected sequences of these variants (Fig. 3D,E). Next, we mixed different amounts of the WT, 1U, and 4U pre-let-7a-1 variants and demonstrated that it was possible to correctly identify all the variants (and in the correct ratios) in these mixtures with iLIME-seq (Supplemental Fig. S3B). These results indicate that iLIME-seq can detect and estimate the proportions of different pre-miRNA variants.

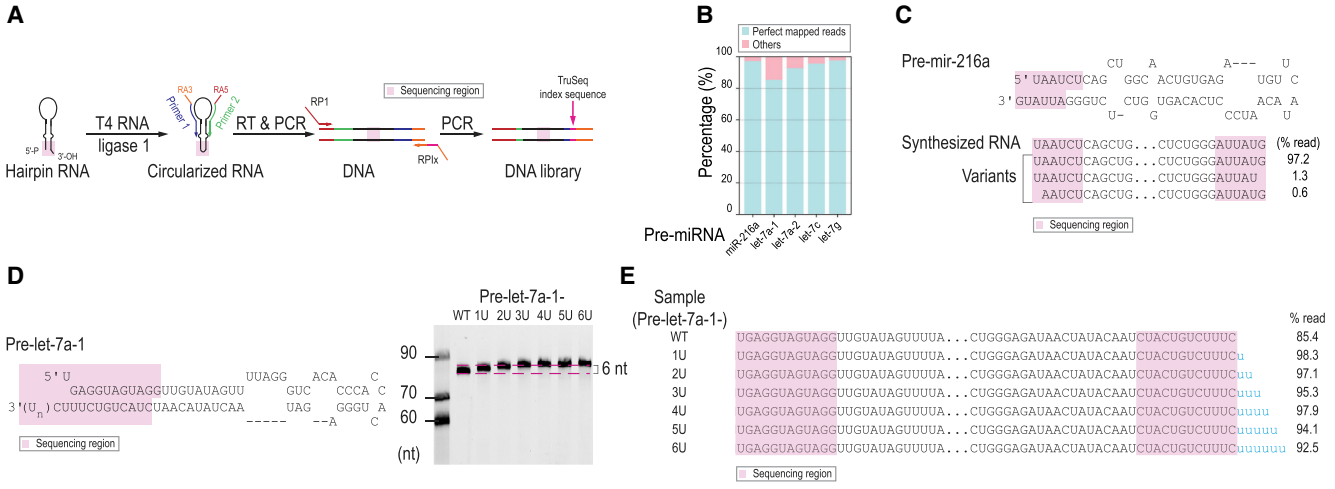


**FIGURE 3.** Sequencing synthesized pre-miRNAs by iLIME-seq. (*A*) The diagram to show iLIME-seq. This is conducted using one RT and two PCR steps. The adapter and sequencing sequences are added in the PCR primers of the first and second PCR steps, respectively. (*B*) iLIME-seq was conducted for synthesized pre-miRNAs, and the reads resulting from NGS were mapped to the pre-miRNAs. The percentages of the perfect mapped reads were estimated for each pre-miRNA. (*C*) The sequencing results obtained after iLIME-seq were conducted for pre-mir-216a. (*D*) The quality of the synthesized uridylated pre-let-7a-1 variants were assessed via 12% urea-PAGE. (*E*) iLIME-seq was conducted on wild-type (WT) and six (1U–6U) uridylated pre-let-7a-1 variants. The percentage of accurate pre-miRNA sequences was estimated from the NGS results. Primer sequences used for pre-miRNA sequencing libraries are shown in Supplemental Table S2.

## Sequencing endogenous pre-miRNAs with iLIME-seq

In one series of experiments, we performed iLIME-seq for 10 pre-miRNAs in two different cell types, HCT116 and HEK293T. Our sequencing data demonstrated that iLIME-seq captured the typical ends of the human pre-miRNAs found in previous studies (Liu et al. 2014; Kim et al. 2015; Treiber et al. 2019; Yu and Kim 2020). They contained three types of pre-miRNAs: (1) Intact pre-miRNAs, which mapped perfectly to pre-miRNAs; (2) elongated pre-miRNAs, which contained a few additional nt at the 3′-end; and (3) trimmed pre-miRNAs, which lost a few nt at the 3′-end (Fig. 4A).

We also showed that iLIME-seq detected isoforms of many pre-miRNAs (Fig. 4B). We named the isoforms using the CLx/y system, such that "CL" stands for "cleavage," "x" is the position of the 5′-end, "y" is the position of the 3′-end of pre-miRNAs. For example, iLIME-seq identified three pre-miR-142 isoforms (namely pre-miR-142_CL-2/-2, CL0/0, and CL+1/+1), which are known to exist in human cells (Fig. 4C,D; Chen et al. 2004; Wu et al. 2009; Ma et al. 2013). In addition, iLIME-seq also detected two major isoforms (CL0/0 and CL+1/+1) of pre-mir-342, which result from the alternative cleavages of DROSHA (Wu et al. 2009; Kim et al. 2017; Li et al. 2020). Interestingly, we also identified a new isoform of pre-mir-342 (CL0/+1) (Fig. 4C,D). This isoform might be another product resulting from the alternative cleavage of DROSHA, or it might be a 1-nt-trimmed product of pre-mir-342_CL0/0. iLIME-seq also detected pre-let-7s and their ac-pre-let-7s, CL0/+11 (Fig. 4B; Supplemental Fig. S4), which resulted from cleavage by AGO (Diederichs and Haber 2007), as well as new isoforms of both pre-let-7a-3, namely CL0/-1 (28%) and CL-1/-1 (2.6%), and pre-let-7g, namely CL-1/-1 (0.8%), which have not been reported before. The accumulation of a particular pre-miRNA variant detected by iLIME-seq might have resulted from the decreased pre-miRNA cleavage of DICER or the increased alternative pri-miRNA cleavage of Microprocessor.

Interestingly, iLIME-seq showed different distributions of the pre-miRNA isoforms for some of the pre-miRNAs when comparing the two cell lines (Fig. 4B). For example, pre-let-7a-1_CL0/0 accounted for 91.4% of all isoforms found in HEK293T cells, whereas its coverage was only

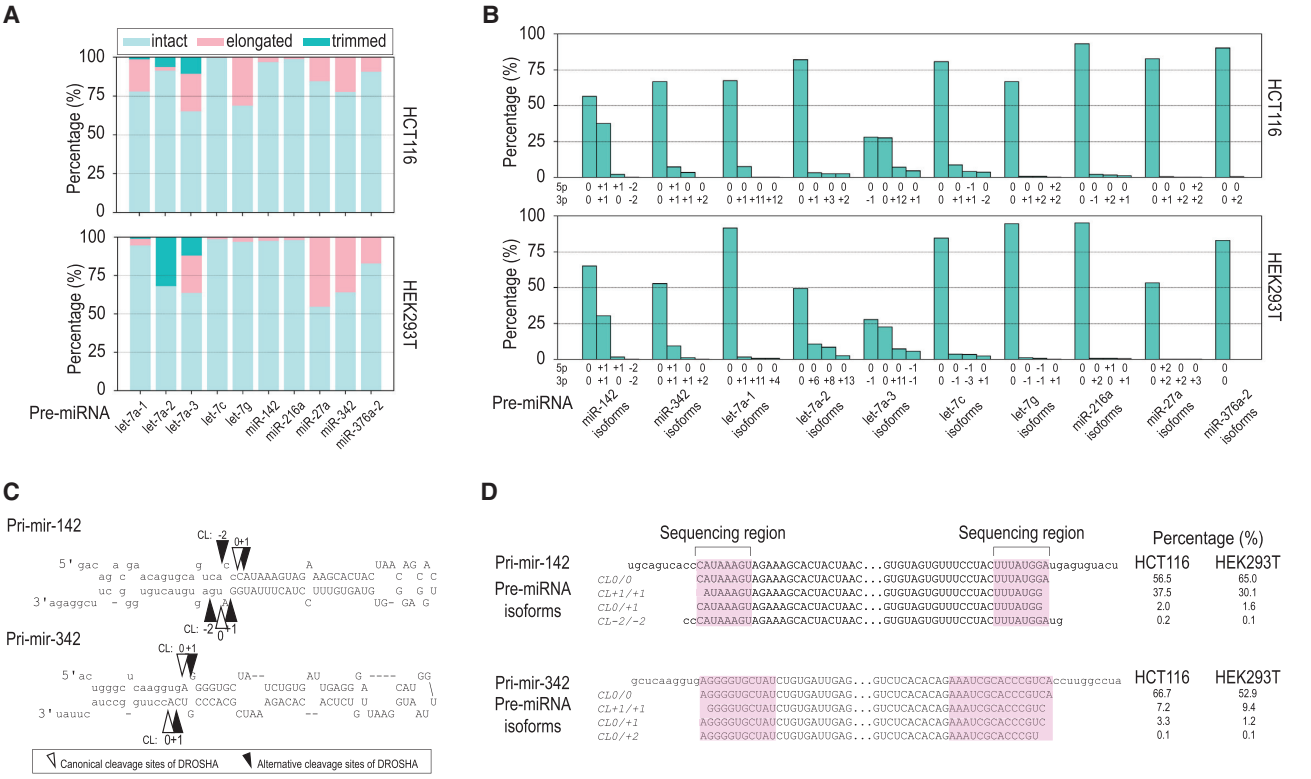


**FIGURE 4.** Sequencing of endogenous pre-miRNAs by iLIME-seq. (*A*) iLIME-seq was conducted for many endogenous pre-miRNAs in HCT116 and HEK293T cells. The sequences resulting from NGS were mapped to pre-miRNAs. Three common types of pre-miRNAs, namely intact, elongated, and trimmed, were identified. (*B*) iLIME-seq revealed different pre-miRNA isoforms. The 5′-end and 3′-end positions of pre-miRNAs are shown. The ac-pre-let-7, Ago2-cleaved pre-let-7, is the cleavage product of pre-let-7 by Ago2 (Diederichs and Haber 2007). (*C*) Diagrams to show pri-miRNAs and the positions of the 5′-end and 3′-end of their corresponding pre-miRNA isoforms. (*D*) The percentages of different pre-mir-142 and pre-mir-342 isoform sequences are shown as representative examples. Primer sequences used for the pre-miRNA sequencing library are shown in Supplemental Table S2.

67.5% in HCT116 cells (Fig. 4B; Supplemental Fig. S4). On the other hand, the percentage of pre-let-7a-1_CL0/+1 was only 1.5% in HEK293T cells, but it was 7.4% in HCT116 cells (Fig. 4B; Supplemental Fig. S4). These data indicate that different cell lines might utilize different pathways for the metabolism of pre-miRNAs.

### iLIME-seq reveals modifications in different pre-miRNAs isoforms

We examined the 3′-end modifications of 10 pre-miRNAs and found that most contain uridine (Fig. 5A,B), so we classified the U-modification levels for each pre-miRNA isoform. Interestingly, iLIME-seq allowed us to isolate different isoforms of pre-miRNAs containing various modifications (Fig. 5A; Supplemental Fig. S5). For example, pre-mir-142_CL-2/-2 contained more U-modifications than the CL0/0 and CL+1/+1 isoforms (Fig. 5C,D). In addition, the CL0/+1 and CL+1/+1 isoforms of pre-mir-342 contained more U-modifications than CL0/0 (Fig. 5C,D). These results indicate that iLIME-seq can allow us to detect the isoforms and modifications of each isoform simultaneously.

## DISCUSSION

We demonstrated that the intramolecular ligation (catalyzed by T4 RNA ligase 1) between the 5′- and 3′-ends of pre-miRNAs is efficient. Typically, we achieved ~80% ligation events for all the pre-miRNAs tested. This might be explained by the fact that the 5′- and 3′-ends of a pre-miRNA are near each other, allowing the T4 RNA ligase 1 to connect them easily. This high intramolecular ligation efficiency might also block the intermolecular ligation of the 5′-end of pre-miRNAs with other RNA molecules, such as 5′-adapters. In classical RNA sequencing, the 3′- and 5′-ends of pre-miRNAs are ligated to 3′- and 5′-adapters, respectively. However, it has been reported that 5′-end ligation is highly inefficient (Burke et al. 2014; Liu et al. 2014; Lama et al. 2019). One explanation might be that the nt at the 5′-end of pre-miRNAs is base-paired with another nucleotide on its opposite strand, which reduces the ligation efficiency. Here, we provide another explanation: The 5′-end of each pre-miRNA molecule is so efficiently intramolecularly ligated to its 3′-end that it cannot be ligated efficiently to the 5′-adapter.

Pre-miRNAs are a central component of miRNA biogenesis (Ha and Kim 2014; Bartel 2018). They are the products of pri-miRNA processing and the precursors of miRNAs. Their expression levels reflect the activity of pri-miRNA cleavage in upstream steps and determine miRNA expression levels downstream. Therefore, it is important to be able to quantify pre-miRNAs in order to gain a better understanding of miRNA biogenesis. This is especially important as many miRNAs have been shown to be useful biomarkers for a plethora of human diseases (Condrat et al. 2020). Although pre-miRNAs are as crucial as miRNAs, to date, their use as biomarkers has been limited. One of the reasons for this is the lack of accurate and sensitive pre-miRNA quantification methods. Indeed, the quantification of pre-miRNAs has been a challenge

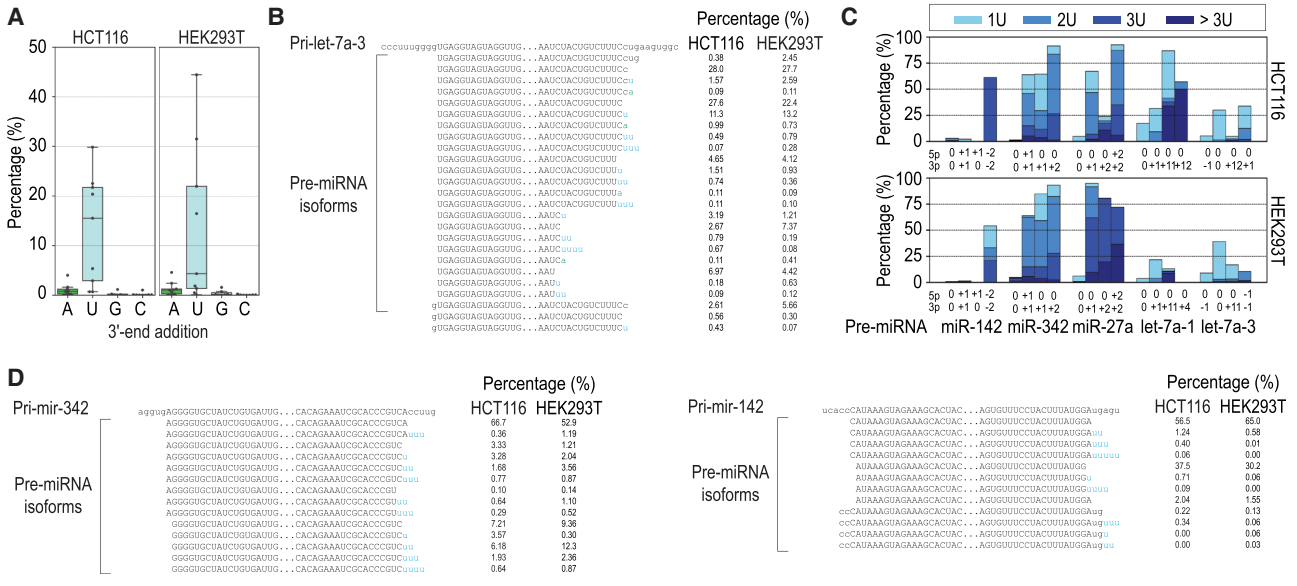


**FIGURE 5.** iLIME-seq detects 3′-end modifications in different pre-miRNA isoforms. (*A*) iLIME-seq detects different modifications at the 3′-end of endogenous pre-miRNAs. The four types of modifications (A, U, G, and C) at the 3′-end of 10 pre-miRNAs (shown in Fig. 4) were sequenced by iLIME-seq. (*B*) Modifications at the 3′-end of pre-let-7a-3 isoforms are shown as a representative example. (*C*) The 3′-end uridylation of different pre-miRNA isoforms. The percentage of uridylation (including mono-uridylation, di-uridylation, triuridylation, and oligouridylation), was estimated for each pre-miRNA isoform. (*D*) The 3′-end uridylation of pre-mir-142 and pre-mir-342 isoforms is shown as representative examples. Primer sequences used for the pre-miRNA sequencing library are shown in Supplemental Table S2.

because their structure is also held within pri-miRNAs and so classical qPCR quantifies both pre-miRNAs and their parental pri-miRNAs simultaneously. The iLIME-qPCR can amplify (and thus quantify) pre-miRNAs exclusively, and our hope is that this facilitates future research into the use of pre-miRNAs as biomarkers. Furthermore, we suggest that this methodology can be used to readdress many previous pre-miRNA quantification findings obtained using classical qPCR.

Although the iLIME-seq cannot sequence the full-length of pre-miRNAs as other methods (Liu et al. 2014), it can sequence their 5′- and 3′-ends, where various function-related modifications and changes occur in pre-miRNAs (Liu et al. 2014; Kim et al. 2015; Treiber et al. 2019; Yu and Kim 2020). The iLIME-seq uses a simple and convenient way to clone pre-miRNAs of interest for sequencing their ends. The DNA libraries can be made within a day, and they require a few steps and simple reagents (including T4 RNA ligase 1 and common PCR reagents). In addition, the iLIME-seq does not need the 5′-end and 3′-end adapter ligations, which have low efficiency (particularly for pre-miRNAs) and are time-consuming to achieve. Therefore, using iLIME-seq, we can obtain information about the ends of pre-miRNAs, which will be useful for various investigations. Indeed, we already demonstrated that iLIME can detect pre-miRNA isoforms, pre-miRNA modifications at 3′-ends, and a combination of isoforms or modifications. miRNA isoforms (isomiRs) can be produced in human cells by multiple mechanisms. For example, mature miRNAs are trimmed or added with one or few nt (Yu and Kim 2020). In addition, pre-miRNA isoforms, which are resulted from the alternative cleavages of Microprocessor in pri-miRNAs or from nt addition or trimming at their ends, are cleaved by DICER, and thus miRNA isoforms are generated (Bartel 2018). It is essential to discriminate isomiRs resulting from DICER cleavage or modifications at the mature miRNA stage. The ability of iLIME-seq to sequence the ends of pre-miRNAs in combination with the miRNA sequencing method would be helpful to address this question.

In addition to pre-miRNAs, cells also contain many other types of RNAs (including tRNAs, snRNAs, and snoRNAs), which contain hairpin structures. These RNAs are also produced from their longer precursors, and so (like pre-miRNAs), it is difficult to discriminate them from their parental precursors. The 5′- and 3′-ends of many of these hairpin-containing RNAs are also situated near to each other, so it is possible that iLIME-qPCR and iLIME-seq can be extended to respectively quantify and sequence these RNAs.

## MATERIALS AND METHODS

### RNA synthesis

The double-stranded DNA (dsDNA), containing the T7 promoter, the hammerhead (HH) ribozyme, a pre-miRNA sequence, and the hepatitis delta virus (HDV) ribozyme, was synthesized by one step of the oligo extension and two sequential PCRs. In the oligo extension step, two oligos containing the T7-HH ribozyme and pre-miRNA sequences were partially annealed and then extended for 2 h at 37°C using the Klenow Fragment (Thermo Fisher Scientific, EP0421), resulting in a complete dsDNA. In the first PCR, the dsDNA from the previous extension step was amplified and extended using the T7 primer (T7-GGG, Supplemental Table S1) and the first PCR reverse primer containing the 5′ half of HDV ribozyme sequence. The HDV-dsDNA, including the sequence of the HDV ribozyme, was prepared from a separate PCR with three oligos. The second PCR was an overlapped PCR. The first PCR DNAs and HDV-dsDNA, which shared the 5′ half of the HDV ribozyme sequence, were amplified using the T7 primer and the second PCR reverse primer containing the 3′ half of HDV ribozyme sequence (R-HDV ribozyme, Supplemental Table S1). All the PCR reactions used Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, F549L), and all oligos used above were obtained from GENEWIZ and the BGI Group.

Two hundred nanograms of each dsDNA template was added to the IVT reaction containing the reagents provided in the MEGAscript T7 Transcription Kit (Invitrogen). The resulting RNAs were self-cleaved by the activity of the HH and HDV ribozymes, releasing the pre-miRNA containing -OH and 2′–3′-cyclic phosphate at its 5′- and 3′-ends, respectively. The pre-miRNA was gel-purified. Subsequently, 2′–3′-cyclic phosphate and 5′-OH in the pre-miRNA were converted into 3′-OH and 5′-Phosphate, respectively, by T4 polynucleotide kinase (Thermo Fisher Scientific, EK0032), as described in our previous study (Nguyen et al. 2022). The 5′-phosphate pre-miRNAs were purified using isopropanol and stored at −80°C until further use.

The pri-miRNA synthesis was conducted in a similar way as described in our previous studies (Li et al. 2020; Nguyen et al. 2020). The oligo sequences used in the RNA synthesis are shown in Supplemental Table S1.

### Pre-miRNA ligation

Five hundred nanograms of total RNAs (with 0.01 fmol spike-in RNA added) or two pmol of synthesized pre-miRNAs were ligated by 16 units of T4 RNA ligase 1 (NEB, M0437M) in 16 µL NEB T4 RNA ligase reaction buffer (NEB, M0204S), comprising 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT. Sixteen units of SUPERase•In RNase inhibitor (Thermo Fisher Scientific, AM2694) and 20% PEG 8000 were then added to the ligation reaction. The ligated mixtures were then treated with 10 units RNase R.

### iLIME-qPCR

Five hundred nanograms of total RNA (with 0.01 fmol spike-in RNA added) or different amounts of pre-miRNAs were ligated by T4 RNA ligase 1 to generate circularized pre-miRNAs. The ligation reactions were then added to five units of RNase R and incubated at 45°C for 1 h before conducting reverse-transcription by Superscript IV (Invitrogen, 18090010) and RT primers. The RT primers form ∼10 and 15 bp with the loop and stem of pre-miRNAs, respectively. In addition, a nucleotide at the 3′-end of the primers makes a base pair with a mismatched nucleotide in

the stem of pre-miRNAs. The resulting cDNAs were incubated with 10 units of RNase H for 1 h and amplified using a pair of PCR primers in the PCR reaction containing SYBR Green I (Thermo Fisher Scientific, S7563) and Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific, F549L). The PCR products were real-time quantified using a Roche LightCycler 480 Real-time PCR system. The oligo sequences used in iLIME-qPCR for pre-miRNAs and in qPCR for pri-miRNAs are shown in Supplemental Tables S2 and S3, respectively. The raw results of iLIME-qPCR and qPCR are shown in Supplemental Table S4–8.

### iLIME-seq

Two hundred and fifty fmol pre-miRNAs or 2500 ng of total RNA were ligated with T4 RNA ligase 1. The ligated pre-miRNAs were then reverse-transcribed by Superscripts IV (Invitrogen, 18090010) and RT primers formed 20–30 bp with pre-miRNAs. The reverse complementary sequence of the 3′-adapter (5′-TGGAATTCTCGGGTGCCAAGG-3′) was included in the RT primers. The resulting cDNAs were first amplified using the reverse primer (5′-CCTTGGCACCCGAGAATTCCA-3′) and the forward primer containing the 5′-adapter sequence (5′-GTTCAGAGTTCT ACAGTCCGACGATC-3′) before being amplified using RPIx and RP1 primers to generate the DNA libraries. The DNA libraries were sequenced using an Illumina Nextseq 500 sequencer using a Nextseq Mid Output (2 × 75) cycles kit. The oligo sequences used in the iLIME-seq are shown in Supplemental Table S2.

### iLIME-seq analysis

The resulting DNA sequences obtained from NGS were treated with cutadapt (Martin 2011) (cutadapt -a TGG AAT TCT CGG GTG CCA AGG -A GAT CGT CGG ACT GTA GAA CTC TGA AC) to remove the adapter sequences. The trimmed paired-end sequencing reads were then joined using fastq-join (with default parameters). Low quality reads were discarded using fastq_quality_filter (-q 30 -p 90) (FASTX-Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/index.html, version 0.0.13). The remaining sequencing reads were used for analyzing pre-miRNA isoforms and modifications using the following pipeline. In brief, two regions of sequencing reads, complementary to two PCR primers of the first PCR in the library construction, were mapped to the pre-miRNA sequences, assigning the mapped reads pre-miRNAs. Subsequently, the unmatched regions of the sequencing reads were further mapped to the assigned pre-miRNAs. This second mapping step determined the terminal 5′- and 3′-ends of the reads that could be mapped to the pre-miRNAs, classifying the reads into three categories: intact, trimmed, and elongated pre-miRNAs. The inserted region of the elongated pre-miRNAs was further analyzed to identify the nucleotide sequence of the insertion. The sequencing region between two ends of the pre-miRNA isoforms was then used for checking modifications. The raw data of NGS can be found at GEO: GSE185524.

### Cell culture and total RNA isolation

HCT116, DROSHA-KO, and DICER-KO cells were cultured in McCoy's 5A medium (Gibco, 16600082), and HEK293T cells were cultured in DMEM medium (Gibco, 12100061). These two media were supplemented with 10% fetal bovine serum (FBS) (Gibco, 10270106) and 1% penicillin-streptomycin (Gibco, 15140122). The cells were cultured in a 100 mm dish until they reached 80% confluency, at which time the total RNA was isolated from the cells using 800 µL TRIzol reagent (Invitrogen, 15596018).

## DATA DEPOSITION

NGS data can be found at GEO: GSE185524.

## SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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*Author contributions:* M.N.L. and T.A.N. designed the experiments. M.N.L. conducted the experiments. C.T.L. performed data analysis. M.N.L., C.T.L., and T.A.N. interpreted the results and wrote the manuscript.

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*See the following page for* ***Meet the First Author***

## MEET THE FIRST AUTHOR

Minh Ngoc Le

***Meet the First Author(s)* is a new editorial feature within *RNA*, in which the first author(s) of research-based papers in each issue have the opportunity to introduce themselves and their work to readers of *RNA* and the RNA research community. Minh Ngoc Le is the first author of this paper, "Intramolecular ligation method (iLIME) for pre-miRNA quantification and sequencing." Minh Ngoc is an MPhil-PhD student at The Hong Kong University of Science and Technology who is interested in studying miRNA biogenesis and RNA-binding proteins.**

**What are the major results described in your paper and how do they impact this branch of the field?**

This paper developed a method called iLIME-qPCR and iLIME-seq to quantify and sequence pre-miRNAs specifically. In this method, we utilized the T4 RNA ligase 1 to circularize pre-miRNAs, allowing us to design primers that can only amplify pre-miRNAs, not pri-miRNAs. Thus, our developed iLIME-qPCR can quantify pre-miRNAs precisely, advancing the classical qPCR that quantifies pre-miRNAs and pri-miRNAs simultaneously. In addition, the PCR products that result from the circularized pre-miRNAs are converted into DNA libraries for sequencing. An additional advantage of iLIME is that it provides a simple and effective way to sequence the 5′- and 3′-ends of pre-miRNAs.

We believe that our iLIME method will benefit the RNA community in multiple ways. (1) iLIME-qPCR is a unique but effortless way to quantify pre-miRNAs, so we hope it will encourage more researchers to investigate the possibility of utilizing pre-miRNAs as biomarkers. iLIME-qPCR provides an efficient method for quantifying pre-miRNAs to address various questions related to miRNA biogenesis. (2) In addition, using iLIME-qPCR will help correct the results of previous research studies that used commercial kits to quantify pre-miRNAs. As mentioned above, this kit quantifies both pre-miRNAs and their parental pri-miRNAs and, therefore, cannot differentiate between them. (3) iLIME-seq also provides a practical and straightforward way to sequence the ends of pre-miRNAs and, therefore, can be used to identify the isoforms and end modifications of pre-miRNAs, which are essential for miRNA biogenesis and function.

**What led you to study RNA or this aspect of RNA science?**

In the fourth year of my undergraduate studies, one of my professors excitedly shared with me the news that the FDA just approved an antisense oligonucleotide (ASO) drug, called Nusinersen (Spinraza), to treat spinal muscular atrophy, a fatal genetic disease that affects children and adults. I started to study more about ASOs and their mechanisms as well as their medical applications. I realized that RNAs have great potential in research and clinical applications. Among many types of RNAs, I especially liked to read about miRNAs, which have been reported as potential biomarkers and drugs for treating many diseases. Therefore, I chose to join the RNA group of Professor Nguyen at HKUST to study miRNAs further.

**What are some of the landmark moments that provoked your interest in science or your development as a scientist?**

I started a dream to become a scientist when, at the age of 12, I visited the university where my dad worked. At that time, I had an opportunity to observe my dad's students conducting experiments. I saw them mixing some transparent chemical solutions to obtain plenty of colorful solutions. I was totally amazed at what I had seen and have felt in love with the experiments since then. I entered the university many years later and could perform as many experiments as I wished to. I reaffirmed my passion for science and determined to become a great scientist.

**What are your subsequent near- or long-term career plans?**

Briefly, I would still like to study more about miRNAs. I want to use the iLIME that we developed to understand more miRNA biogenesis. In addition, I also wish to investigate the possibility of using iLIME to quantify and sequence pre-miRNAs from clinical samples, which may reveal differences in pre-miRNA expression and sequences between disease and control tissues. In my long-term career plans, I hope I will have a chance to learn more about RNA metabolism in human diseases.