

**DynaExpress miRNA Cloning Kit High Efficient**

**Product Name :** DynaExpress miRNA Cloning Kit High Efficient

**Code No. :** DS330

## PRODUCT INFORMATION

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### Background

Several classes of small non-coding RNAs have been found and are studied. Small interfering RNA (siRNA) and microRNA (miRNA) are two major classes of these small non-coding RNAs. In the breakthrough finding known as RNA interference (RNAi), small duplex RNA, that is, siRNA, plays a key role in gene silencing (1). The siRNA combines with cellular proteins to construct an RNA-induced silencing complex (RISC), which leads to cleavage of a targeted messenger RNA (2). miRNAs are regulatory molecules and the mature form is a single-stranded, 18-28 nucleotide RNA. The first miRNA, lin-4, which was called small temporal RNA at that time, was discovered in 1993 (3). The gene of lin-4 does not have ORF but was found to encode a small RNA, which represses the translation of a transcription factor gene, lin-14. The term, microRNA (miRNA), was introduced in 2001 in Science papers (4-6). miRNAs have a complementary portion to one or more mRNA(s). Plant miRNAs are in most cases perfectly complementary to their target mRNAs, in contrast, most animal miRNAs exhibit imperfect complementarities to mRNA sequences within 3'UTRs (7). After binding to mRNA, some miRNAs inhibit protein translation and some of them cleave mRNA. It seems to be dependent on miRNA's complementarity to mRNA (8). The biogenesis of miRNA proceeds through the formation of two precursors. The primary structure of miRNA, called pri-miRNA (several kilobase), is transcribed from genes of miRNAs. In animals, the pri-miRNA are processed by RNase III enzyme, Drosha, to produce a hairpin intermediate, pre-miRNA (60 - 100 nucleotides) in a nucleus (9). The pre-miRNA is transported to cytoplasm by Exportin 5, which is known as the tRNA exporter (10), and diced by Dicer to form a mature miRNA structure. The plant miRNA biogenesis is somewhat different from animal's (11). In plants, Dcl I, RNase III enzyme, processes an mRNA precursors to its mature form. miRNA is exported to cytoplasm after being matured. Dicer is also known as the RNase III enzyme involved in generation of siRNA(12). siRNA is generated through a similar pathway of miRNA. After cleavage of pre-miRNA by Dicer, only one strand of the miRNA duplex is integrated to miRNP/miRISC complex as a guide RNA. The miRNP/miRISC complex, which contains a matured single stranded miRNA and a member of the Argonaute protein family, is a functional unit for regulation of gene expression (13). Due to a great deal of studies on miRNAs, it has been evident that miRNAs act as key regulatory molecules in apoptosis (14), development (15), viral infection (16) and others (17, 18).

In order to determine functions of siRNA, miRNA and other small non-coding RNAs, cloning and sequencing of these RNA molecules is a fundamental and indispensable study. This product was designed from knowledge of published reports (4-6, 20, 21). Using the <sup>DynaExpress</sup> miRNA Cloning Kit High Efficient, research will make progress exploring new biological findings.

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### Kit Contents and Storage

• This kit provides reagents performing for five reactions of miRNA cloning and for two fractionations of miRNA from total RNA (See Additional Information).

• These Boxes (Box 1 and 2) are shipped on dry ice. Upon receipt, store each box as below.

### Kit contents

#### Box 1: Store at -20°C (Do not store in frost-free freezer)

	Component	Description	Amount	Storage
1	MI-A3' Linker		7 µl	- 20°C
2	MI-R5' Linker		7 µl	- 20°C
3	Control RNA (21 mer)	p5' RNA, ca. 0.025 µg/µl	10 µl	- 20°C
4	Ligase 3'		7 µl	- 20°C
5	Ligase 3' Buffer (10 ×)		45 µl	- 20°C
6	Ligase 5'		7 µl	- 20°C
7	Ligase 5' Buffer (10 ×)		45 µl	- 20°C
8	RNA Loading buffer PA	DM156 (90% formamide‡)	1 ml	- 20°C
9	Colored Guide Size Marker		150 µl	- 20°C
10	PEG Solution	45% polyethylene glycol	150 µl	- 20°C
11	RT Buffer (5 ×)		45 µl	- 20°C
12	10 mM dNTPs Mixture	10 mM each dNTP	20 µl	- 20°C
13	Reverse Transcriptase	100 u/µl	7 µl	- 20°C
14	Taq DNA Polymerase	5 u/µl	7 µl	- 20°C
15	Amplification Buffer (10 ×)		80 µl	- 20°C

‡ Formamide is suspected to be harmful. It is an irritant to the eyes and skin. Wear appropriate gloves and safety glasses. Put a lid on tightly at the time of storage.

#### Box 2: Store at -20°C (Do not store in frost-free freezer)

	Component	Description	Amount	Storage
1	Nuclease-Free Water		1.6 ml × 4	- 20°C
2	3' RT Primer	25 µM	20 µl	- 20°C
3	5' Primer	25 µM	20 µl	- 20°C
4	Glycogen	5 µg/µl	150 µl	- 20°C
5	Sodium Acetate Solution	3 M	1 ml	- 20°C
6	RNA Extraction Buffer		10 ml	- 20°C

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### Compositions of reaction buffers

Buffer	Composition
RT Buffer (5 ×)	250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl <sub>2</sub> , 50mM DTT
Amplification Buffer (10 ×)	200 mM Tris-HCl (pH8.8), 100 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 100 mM KCl, 20 mM MgSO <sub>4</sub> , 1% Triton X-100
RNA Extraction Buffer	20 mM Tris-HCl, (pH 8), 0.1 mM EDTA, 0.3 M NaCl, 0.1% SDS

### Nucleotide Sequence

Name	Sequence	
MI-A3' Linker	5' AppCTGTAACCTCGGGTCAATddC	DNA
MI-R5' Linker	5' AUCGUAGGCACCUGACC	RNA
3' RT Primer	5' ATTGACCCGAGTTACAG	DNA
5' Primer	5' ATCGTAGGCACCTGACC	DNA
Control RNA (21 mer)	5' pUGAGGUAGUACUCAAGUGUGA	RNA

### About Colored Guide Size Marker

The Colored Guide Size Marker is designed for the purpose of fractionating 18-28 mer RNAs and their MI-A3' Linker ligated products, as well as their MI-A3' Linker and MI-R5' Linker ligated products. Colored Guide Size Marker consists of six pre-stained oligo nucleic acids, I, II, III, IV, V, VI. (See Fig.2). The apparent size of band I and II correspond to that of 5'-phosphorylated 18 and 28 mer RNAs, respectively on denaturing polyacrylamide electrophoresis. The apparent size of band III and IV correspond to that of MI-A3' Linker ligated products (36, 46 mer) on denaturing polyacrylamide electrophoresis. Likewise, band V and VI correspond to MI-A3' Linker and MI-R5' Linker ligated products (53, 63 mer).

### Notes on handling of RNA

RNase is a very powerful enzyme that degrades RNA. To avoid damaging RNA and ligation products containing RNA, use extreme care during manipulations to prevent nuclease contamination. Wear gloves and use clean apparatus. Nuclease-free disposable plasticware should be used. To set up polyacrylamide gel electrophoresis apparatus, just use clean and dry chamber and glass plates rinsed with autoclaved distilled water or MilliQ water. Use a clean razor blade for gel excision. DEPC treatments is not necessary for electrophoresis chamber, glass plates and a gel tray.

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### Additional Materials and Equipment Needed

- TE-saturated phenol (pH 7.8 - 8.0)
- Chloroform
- Ethanol (absolute)
- 1 × TBE (Tris/Borate/EDTA buffer)
- Polyacrylamide gel (10% and 15%)
- Sterile 1.5 ml microfuge tubes
- Sterile 0.5 ml microfuge tubes
- Clean razor blade
- Micropipet
- Micropipet tips
- Apparatus for polyacrylamide gel electrophoresis
- Power supply
- Microcentrifuge
- Thermal cycler for PCR
- 1 × TAE (Tris/Acetate/EDTA buffer)
- Agarose
- Bunsen burner
- Ice bucket
- Vortex mixer
- Shaker or agitator
- T vector is not provided in the kit.

### Related Products

DS315	miRNA fractionation Kit
DS120	DynaExpress <sup>™</sup> TA PCR Cloning Kit, with Jet Competent Cell
DS125	DynaExpress <sup>™</sup> TA PCR Cloning Kit
DS210	Competent Cell JM109
DS220	Competent Cell DH5 $\alpha$
DS225	Jet Competent Cell (DH5 $\alpha$ )

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### Procedure of miRNA cloning

#### Experiment Outline

The <sup>DynaExpress</sup> miRNA Cloning Kit High Efficient is the kit for the cloning of mature miRNAs, siRNAs or other small RNAs of 18-28 nucleotides. In the first step, miRNAs are directly ligated to an activated oligonucleotide, MI-A3' Linker, and form a 5'-phosphorylated and 3'-terminal blocked nucleotide. Dephosphorylation of miRNAs is not required. In the reaction, ATP is not included in the reaction mixture so self ligation or circularization of the miRNAs does not happen. The following polyacrylamide gel electrophoresis successfully separates the MI-A3' Linker ligated products and remaining MI-A3' Linker. In this separation, Colored Guide Size Marker clearly indicates the portion to be excised on the gel. In the second step, the 5'-phosphate end of the miRNA is ligated to MI-R5' Linker. The blocked 3'-end with the MI-A3' Linker also prevents circularization on the second step. The two-linkers ligated products are purified by polyacrylamide gel electrophoresis according to the indication of Colored Guide Size Marker. The resulting product, MI-R5' Linker + miRNA + MI-A3' Linker, is reverse transcribed and then amplified by PCR. The resulting cDNA is ready for cloning into a T vector.

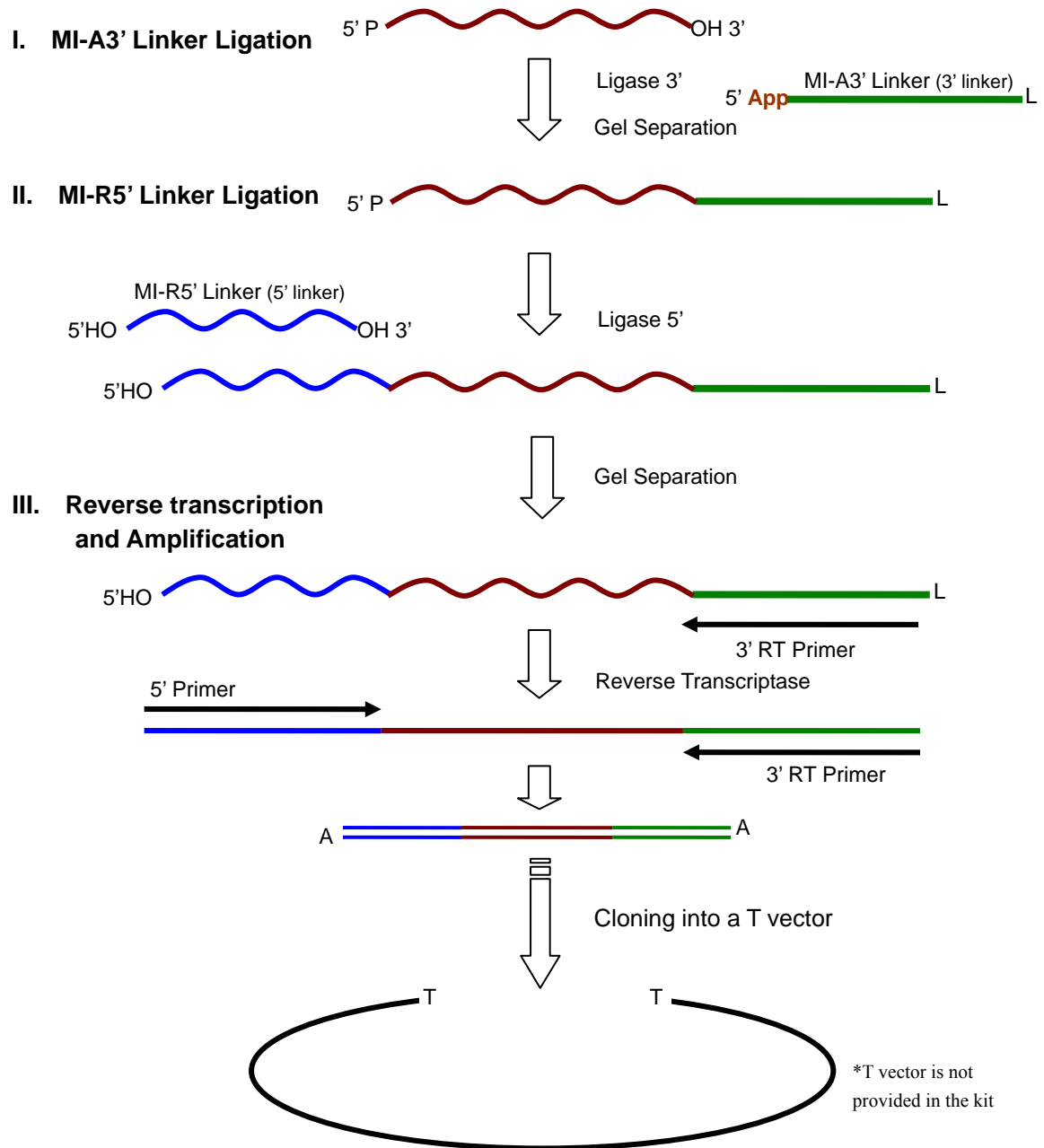
#### Protocol

- I. MI-A3' Linker Ligation
  1. Ligation reaction
  2. Phenol/Chloroform Extraction and Ethanol precipitation
  3. Purification of MI-A3' Linker ligated product by polyacrylamide gel electrophoresis
- II. MI-R5' Linker Ligation
  1. Ligation reaction
  2. Phenol/Chloroform Extraction and Ethanol precipitation
  3. Purification of two-linkers ligated product by polyacrylamide gel electrophoresis
- III. Reverse Transcription and Amplification
  1. Reverse transcription
  2. PCR Amplification
  3. Gel Electrophoresis

The cloning procedure is divided into 3 parts as above. A section of Protocol in Detail (see the next pages) describes the miRNA cloning method precisely.

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### miRNA cloning scheme (Fig. 1)



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### Protocol in Detail

#### I. MI-A3' Linker Ligation

The <sup>DynaExpress</sup> miRNA Cloning Kit High Efficient requires approximately 5 - 100 ng of miRNA. Roughly speaking, possibly one ng of miRNA is recovered per 10 µg of total RNA. We recommend using at least 100 - 500 µg of total RNA as miRNA source material.

##### 1. Ligation reaction

Set up the following 20 µl reaction in a 1.5 ml of nuclease-free tube and incubate at room temperature (20-25°C) for two hours. In the reaction, MI-A3' Linker is ligated to the small RNA (18-28 nt in length). The ligated product has a blocked 3'-end, which prevents circularization on the MI-R5' Linker ligation step.

Small RNA fraction*	5-100 ng
MI-A3' Linker	1 µl
Ligase 3' Buffer (10 ×)	2 µl
PEG Solution ‡	6 µl
Nuclease-Free Water	add to a final volume of 19 µl
<u>Ligase 3'</u>	<u>1 µl</u>
Total volume	20 µl

Small RNA fraction\*: mature miRNA, siRNA or other small RNA of approximately 18-28 nucleotides.

It is not total RNA.

Notice ‡: PEG Solution is highly viscous. To transfer the solution with a micropipette, slowly take the solution and be sure you picked up the solution you are measuring, and put the solution into a tube slowly, as well. Mix reaction mixture by brief vortexing or pipetting several times.

##### 2. Phenol/Chloroform Extraction and Ethanol precipitation

Add 130 µl of RNase-free Water to the 20 µl reaction and mix briefly. Add 150 µl of TE-saturated phenol to the 150 µl of the solution. Vortex it for 15-30 sec. Centrifuge the tube at top speed (15,000 rpm) for 10 min using a microcentrifuge in the temperature range from 4°C to room temperature. Transfer the supernatant to another nuclease-free 1.5-ml tube. Add 150 µl of chloroform to the supernatant. Vortex it briefly. Centrifuge the tube at top speed for 2 min at 4°C to room temperature. Transfer the supernatant to another nuclease-free 1.5-ml tube. Perform chloroform extraction as above again. Add 15 µl of 3 M NaOAc solution and 3 µl of 5 µg/ml of glycogen. Mix it and centrifuge briefly to collect the contents at the bottom of the tube, then add 500 µl of 100% ethanol to the mixed solution. Put the tube at -80°C for 30 min or more, then centrifuge the tube at top speed for 10 min at 4°C. Remove supernatant. Centrifuge the tube again at top speed for 2 min. Remove the supernatant completely and dry down the precipitate.

##### 3. Purification of MI-A3' Linker ligated product by polyacrylamide gel electrophoresis

In this step, MI-A3' Linker ligated RNA is applied to 10% denaturing polyacrylamide gel electrophoresis and excised from the polyacrylamide gel according to the indication of Colored Guide Size Marker.

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### 3.1. Preparation of 40% acrylamide : bis solution

Acrylamide	190 g
N, N'-methylenebisacrylamide	10 g
<u>H<sub>2</sub>O</u>	<u>to 500 ml</u>

After mixing, filter the solution through a membrane filter (0.45 µm pore size).

### 3.2. Preparation of 10% polyacrylamide /7.5 M urea gel

Prepare urea/acrylamide solution as follows:

40% acrylamide : bis solution	5.0 ml
Urea	9.0 g
10 × TBE	2.0 ml
<u>H<sub>2</sub>O</u>	<u>to 20 ml</u>

After urea is dissolved completely, add 20 µl of TEMED and 160 µl of 10% ammonium persulfate. Mix quickly and then pour the gel into the mold of a vertical gel apparatus (gel size: 7 cm × 8 cm, 1.5 mm thickness, lane width = 5 mm). Let the gel sit until it is solid. The gel apparatus should be assembled according to the manufacturer's protocol and ready to run with 1 × TBE buffer.

### 3.3. Electrophoresis of MI-3' Linker ligated miRNA

Dissolve ethanol-precipitated MI-A3' Linker ligated miRNA with 10 µl of RNA Loading buffer PA.

Heat the MI-A3' Linker ligated miRNA solution for 3 min at 70°C and transfer on ice immediately. Rinse the wells of the gel with 1 × TBE buffer and make sure the lanes are flat for good loading and resolution. Load the entire volume of MI-A3' ligated miRNA onto one center lane. Load 10 µl of Colored Guide Size Marker to another lane next to the lane of the MI-A3' ligated miRNA (Heat treatment is not necessary for Colored Guide Size Marker). It is important to load the MI-A3' Linker ligated miRNA in a similar volume as Colored Guide Size Marker to ensure similar gel running behavior. Electrophorese the gel with a constant voltage of about 150-200 V, until the Bromophenol blue dye reaches a point about 2-3 cm from the bottom.

### 3.4. Extraction of miRNA

After polyacrylamide gel electrophoresis, dismantle the gel-running apparatus. Detach only one of two glass plates from the gel. (Now, the gel is on the other glass plate.) Transfer the gel on the glass plate to the surface of a clean white tray. Compare the lane of Colored Guide Size

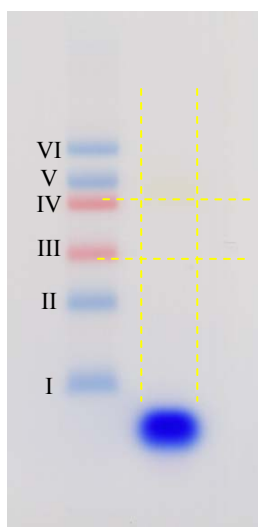


Fig. 2 Excision of MI-A3' Linker ligated miRNA

miRNAs ligated with MI-A3' Linker are 36 - 46 mer RNA/DNA chimera nucleotides. They migrate between bands III (red) and IV (red) of Colored Guide Size Marker on a denaturing polyacrylamide gel (10%) electrophoresis.

The rectangle surrounded by broken yellow lines represents the portion to be excised.

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Marker and the lane of MI-A3' Linker ligated miRNA, and cut out the gel portion in the lane of MI-A3' Linker ligated miRNA corresponding to band III to IV of Colored Guide Size Marker (see Fig. 2). Band III and band IV of Colored Guide Size Marker correspond to 18 mer RNA + 18 mer DNA and 28 mer RNA + 18 mer DNA, respectively. Use a clean razor blade for excision. Transfer the excised gel to a nuclease-free 1.5-ml tube and weight the gel. Heat a clean 200  $\mu$ l-pipette tip in a flame quickly to make round its end and cool it down. Use the pipette tip as a pestle and grind the gel completely. Add 4 volumes of RNA Extraction Buffer. Elute the RNA from the gel by constant agitation at room temperature for approximately 30 min. Centrifuge the tube at top speed (15,000 rpm) for 10 min using a microcentrifuge to precipitate gel pieces in the temperature range from 4°C to room temperature. Transfer the supernatant to another nuclease-free 1.5-ml tube to remove gel pieces. Add an equal volume of TE-saturated phenol to the supernatant. Vortex it for 15-30 sec, centrifuge the tube at top speed for 10 min at 4°C to room temperature. Transfer the supernatant to another nuclease-free 1.5-ml tube. Add an equal volume of chloroform to the supernatant. Vortex it briefly, centrifuge the tube at top speed for 2 min at 4°C to room temperature. Transfer the supernatant to another nuclease-free 1.5-ml tube. Perform chloroform extraction as above again. Add 1/10 volume of 3 M NaOAc solution and 1/50 volume of 5  $\mu$ g/ml of glycogen to the obtained aqueous solution. Mix it and centrifuge briefly to collect the contents at the bottom of the tube, then add three volumes of 100% ethanol to the solution. Put the tube at -80°C for 30 min or more, then centrifuge the tube at top speed for 10 min at 4°C. Remove supernatant. Centrifuge the tube again at top speed for 2 min. Remove the supernatant completely and dry down the precipitate.

## II. MI-R5' Linker Ligation

In this step, MI-R5' Linker is ligated to the 5'-phosphate end of MI-A3' Linker ligated miRNA.

### 1. Ligation reaction

Dissolve precipitate of MI-A3' Linker ligated miRNA with Nuclease-free water in 1.5 ml of tube as below, then set up the following 20  $\mu$ l ligation reaction.

MI-A3' Linker ligated miRNA	precipitate
Nuclease-free water	10 $\mu$ l
MI-R5' Linker	1 $\mu$ l
Ligase 5' Buffer (10 $\times$ )	2 $\mu$ l
PEG Solution	6 $\mu$ l
Ligase 5'	1 $\mu$ l
Total Volume	20 $\mu$ l

Incubate the 20  $\mu$ l reaction at room temperature (20-25°C) for two hours.

### 2. Phenol/Chloroform Extraction and Ethanol precipitation

The 20  $\mu$ l of MI-R5' Linker ligated product is subjected to Phenol/Chloroform Extraction and Ethanol precipitation as above (I. Ligation of MI-3' Linker, 2. Phenol/Chloroform Extraction and Ethanol precipitation).

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### 3. Purification of two-linkers ligated product by polyacrylamide gel electrophoresis

This step is the same as the procedure shown in I. MI-A3' Linker Ligation, 3. Purification of MI-A3' Linker ligated product by polyacrylamide gel electrophoresis. Subject the two-linker ligated miRNAs to denaturing-polyacrylamide gel electrophoresis, and excise gel between band V to VI in Colored Guide Size Marker.

#### 3.1. Electrophoresis of two-linkers ligated miRNA

Dissolve ethanol-precipitated two-linkers ligated miRNA with 10  $\mu$ l of RNA Loading buffer PA and perform electrophoresis. Loading procedure and electrophoresis procedure is the same as the procedures shown in I. MI-A3' Linker Ligation, 3.3. Electrophoresis of MI-A3' Linker ligated miRNA.

#### 3.2. Extraction of two-linker ligated miRNAs

Gel excision procedure is the same as the procedure shown in I. MI-A3' Linker Ligation, 3.4. Extraction of miRNA, but the gel portion to be excised in the lane of the two-linkers ligated miRNA is the corresponding portion between band V and VI of Colored Guide Size Marker (see Fig. 3). Band V and band VI of Colored Guide Size Marker correspond to 63 mer of nucleic acid (46 mer RNA-DNA + 17 mer RNA) and 53 mer of nucleic acid (36 mer RNA-DNA + 17 mer RNA), respectively. Add 4 volumes of RNA Extraction Buffer to the excised gel after grinding as

described in I. MI-A3' Linker Ligation, 3.4. Extraction of miRNA. Elute the RNA from the gel by constant agitation at room temperature for approximately 30 min. The following Phenol/Chloroform extraction and Ethanol precipitation procedure is also the same as in I. MI-A3' Linker Ligation, 3.4. Extraction of miRNA

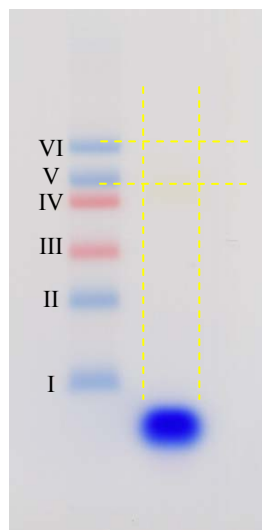


Fig. 3 Excision of MI-R5' Linker ligated miRNA

miRNAs ligated with MI-A3' Linker and MI-R5' Linker are separated by electrophoresis. They migrate between bands V and VI of Colored Guide Size Marker on a denaturing polyacrylamide gel (10%) electrophoresis.

The rectangle surrounded by broken yellow lines represents the portion to be excised.

## III. Reverse transcription and Amplification

This section is the final step in constructing cDNA of miRNA.

### 1. Reverse transcription

Dissolve precipitation of gel purified product with 12  $\mu$ l of Nuclease-free water and transfer it to a 0.5 ml tube. Set up the following 3'-RT primer mixture as follows.

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Gel purified product	12 $\mu$ l
<u>3' RT Primer</u>	<u>1 <math>\mu</math>l</u>
	13 $\mu$ l

Heat the mixture at 70°C for 3 min, cool to room temperature for 2 min, then transfer it on ice.

Set up the following reverse transcription mixture as follow.

Above 3' RT Primer mixture	13 $\mu$ l
RT buffer (5 × )	4 $\mu$ l
10 mM dNTPs Mixture	2 $\mu$ l
<u>Reverse Transcriptase (100 u/<math>\mu</math>l)</u>	<u>1 <math>\mu</math>l</u>
	20 $\mu$ l

Incubate at 42°C for 1 hour, then 90°C for 5 min

### 2. PCR Amplification

In the above reverse transcription, 20  $\mu$ l of cDNA solution is prepared. An aliquot of the cDNA solution is used for amplification in this step. Set up the reaction in a PCR tube (0.2 or 0.5 ml) as described below.

Reverse Transcription Mixture (cDNA)	5.0 $\mu$ l
Nuclease-Free Water	36.5 $\mu$ l
Amplification Buffer (10 ×)	5.0 $\mu$ l
10 mM dNTPs Mixture	0.5 $\mu$ l* <sup>1</sup>
5' Primer	1.0 $\mu$ l
3' RT Primer	1.0 $\mu$ l
<u>Taq DNA Polymerase (5 u/<math>\mu</math>l)</u>	<u>1.0 <math>\mu</math>l</u>
	50.0 $\mu$ l

(0.5  $\mu$ l\*<sup>1</sup> : final conc. is 0.2 mM, because the reverse transcription mixture contains 1 mM dNTP mixture.)

Gently mix the reaction and spin down by microcentrifuge. If the thermocycler does not have a heated cover, add 50  $\mu$ l of mineral oil to the reaction tube to prevent evaporation. Place the PCR tube in the thermal cycler and start the cycling program PCR as below\*<sup>2</sup>.

Initial Heating	94°C for 2 min
20-30 Cycling	94°C for 1 min → 50 °C for 1 min → 72 °C for 1 min
Final Extension	72°C for 10 min

\*<sup>2</sup> Optimum PCR condition varies depending on the thermal cycler used. It is recommended to perform a control experiment to determine the condition. The cycle number depends on the amount of starting small RNA

After cycling amplification is over, PCR product should be checked by 3% agarose gel electrophoresis.

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### 3. Gel Electrophoresis

By PCR, amplified products can be seen. Check the amplified PCR products by 3% agarose electrophoresis according to the standard procedure. Approximately 50-65 bp of products should be seen. The band indicates that cDNA preparation of the miRNA seems to be successful.

Example of agarose gel electrophoresis

- 1) Prepare 3.0% agarose gel in 1× TAE buffer.
- 2) Set the gel electrophoresis apparatus and add sufficient 1× TAE buffer.
- 3) Load 2-5 µl of PCR reaction into a well. Be sure to reserve one well for the DNA size marker, for example, DynaMarker DNA Low D (DM112). Start the electrophoresis.
- 4) After electrophoresis, stain the agarose gel with 0.5 µg/ml ethidium bromide solution, then observe gel on a UV transilluminator.

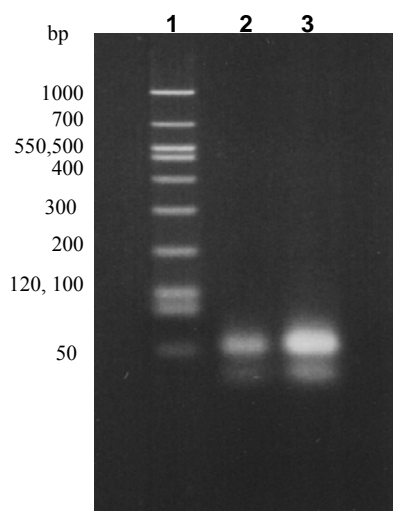


Fig.4 Analysis of PCR Amplification  
miRNA fraction of HL60 total RNA were ligated with MI-A3' Linker and MI-R5' Linker, then subjected to reverse transcription and PCR amplification as described in the Product Information. Obtained PCR product was electrophoresised ( 3 % Agarose, 1×TAE).

Around 60 bp product can be seen, which are from MI-R5' linker + miRNA + MI-A3' linker.

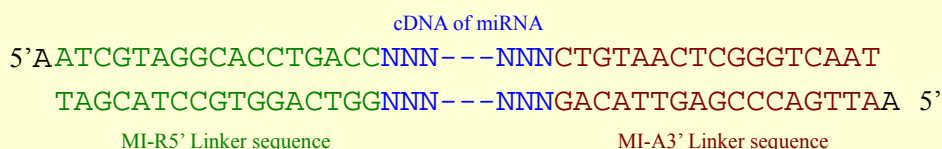
Lane 1: Dyna Marker DNA Low D

Lane 2, 3 : RT-PCR product

Increased volume was loaded into lane 3.

The obtained cDNA, PCR product, can be used for cloning into a T vector, directly (See Fig. 5). Purification of the PCR product, for example gel separation, may work to reduce background on the cloning. If a small amount of miRNA is used in the cloning experiment, after directly cloning into T vectors, some transformants may harbor only linker sequences. Prior to sequencing, colony-PCR or PCR with mini-prep plasmids is helpful to avoid sequencing such a plasmid.

Fig. 5. Structure of cDNA of miRNA



The cDNAs of miRNAs amplified by PCR have MI-R5' Linker sequence and MI-A3' Linker sequence at each end. Taq DNA polymerase adds dA to the 3' end of the cDNA as shown in this figure

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### Additional Information

#### I. Analyze the cDNA of miRNA

To identify whether cloned sequences are of mature miRNA or not, homology searches and other computational approaches are required. Degradated rRNA and tRNA often are contained in the sequences obtained. Tuschl et. al reported that 69 (31%) corresponded to miRNAs, 103 (46%) to already characterized functional RNAs (rRNA, 7SL RNA, tRNAs), 30 (14%) to transposon RNA fragments, and 20 (10%) sequences with no database entry among 225 RNA cloned (4) by basically the same method as that of this kit. First, subject cloned sequences to homology search against miRNA databases, for example, miRBase::Sequences (<http://microrna.sanger.ac.uk/sequences/>). Then, subject cloned sequences to homology analysis against the genome of the organisms used as the RNA source. In this survey, sequences originated from fragments of tRNA or rRNA are removed. The candidates of miRNAs should be matched with the genome. So the genomic regions of the mRNAs also will be found out. Be aware that the lack of a match of sequences may be due to unfinished regions in the genome sequence, sequencing errors, RNA editing (22) or other possibilities (23). The putative miRNA should form a stem-loop structure with the surrounding genomic sequences (that is, miRNA precursors) in the genome. To do it, compute the folding of the sequences with RNA analysis software or m-fold program (<http://www.bioinfo.rpi.edu/applications/mfold/>). A Northern blot experiment is also useful for analysis of the miRNAs. In the experiment, probes are prepared on the base of putative miRNAs sequence and hybridization could be done against total RNA .

#### II. Preparation of Total RNA for small RNA cloning

Small RNA, miRNA/siRNA, is prepared by polyacrylamide gel electrophoresis from total RNA as below. For cloning of miRNA, we recommend using at least 100 - 500 µg of total RNA. You can use total RNA prepared by guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski P. and Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction Anal. Biochem. 162:156-159.) or by commercial total RNA preparation kits. When using a commercial kit, be sure that the kit does not deplete small RNAs. RNA pellet should not be resuspended in 70% of ethanol. Small-size RNA may be easily dissolved in 70% ethanol in the absence of salt. If an ethanol precipitation is performed, you should carefully remove all residual solution and do not need to wash with 70% ethanol.

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### III. Preparation of miRNA by polyacrylamide gel electrophoresis

When total RNA, 100 - 500 µg, is ready, miRNAs, 18-28 nucleotides, are electrophoresed on denaturing polyacrylamide gel. The electrophoresed miRNAs are excised and extracted from polyacrylamide gel on the basis of mobility of Colored Guide Size Marker.

#### 1. Preparation of 15% polyacrylamide /7.5 M urea gel

Prepare urea mixed acrylamide solution as follows:

40% acrylamide : bis solution	7.5 ml
Urea	9.0 g
10 × TBE	2.0 ml
H <sub>2</sub> O	to 20 ml

After urea is dissolved completely, add 20 µl of TEMED and 160 µl of 10% ammonium persulfate. Mix quickly and then pour the gel into the mold of a vertical gel apparatus (7 cm × 8 cm, 1.5 mm thickness, lane width = 12 mm). Let the gel sit until it is solid. The gel apparatus should be assembled according to the manufacturer's protocol and ready to run with 1 × TBE buffer.

#### 2. Electrophoresis of total RNA

Total RNA, 100-500 µg, is ethanol precipitated and dissolved with a small amount of nuclease-free water and RNA Loading buffer PA as indicated in Table 1. Colored Guide Size Marker, 20 µl, is also mixed with 10 µl of RNA Loading buffer PA.

Table 1 Electrophoresis of total RNA

	RNA		Colored Guide Size Marker
	100 - 250	200 - 500	
Total RNA* <sup>1</sup> (µg)	100 - 250	200 - 500	20 µl
Nuclease-Free Water (µl)	15	30	0 µl
RNA Loading buffer PA (µl)	15	30	10 µl
Total volume (µl)	30	60	30 µl
Lane(s)* <sup>2</sup> to load	1 lane	2 lanes	1 lane

Total RNA\*<sup>1</sup>: ethanol precipitate

Lane(s)\*<sup>2</sup>: 1.5 mm thickness, 12 mm width

RNA should be completely dissolved by gentle mixing. If RNA solution is not completely dissolved, the volume may increase slightly by adding RNA Loading buffer PA. In this case, adjust the loading volume of Colored Guide Size Marker solution to that of RNA solution. It is important to load an equal or nearly equal volume of solution to a lane to ensure similar gel running behavior. Heat the prepared RNA solution for 3 min at 70°C and transfer on ice immediately (Heat treatment is not necessary for Colored Guide Size Marker). Rinse the wells of the gel with 1 × TBE and make sure the lanes are flat for good loading and

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resolution. Load the RNA in a lane (or lanes, see Table 1). Load the prepared 30  $\mu$ l of Colored Guide Size Marker solution to another lane next to the lane of the total RNA. Electrophorese the gel with a constant voltage of about 150-200V, until the Bromophenol blue dye reaches a point about 2-3 cm from the bottom.

### 3. Extraction of miRNA

After polyacrylamide gel electrophoresis, dismantle the gel-running apparatus. Detach only one of two glass plates from gel. (Now, the gel is on the other glass plate.) Transfer the gel on the glass plate to the surface of a clean white tray. Compare the lane of Colored Guide Size Marker and the lane of total RNA, and cut out the gel in the lane of sample RNA corresponding to between band I and II of Colored Guide Size Marker (see Fig. 5). Band I and band II of Colored Guide Size Marker correspond to 18 and 28 nt RNA, respectively. Use

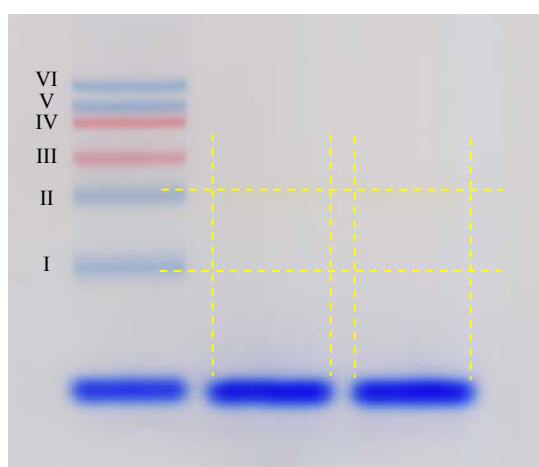


Fig. 5 Excision of 18 - 28 mer small RNAs.

Colored Guide Size Marker (left lane, 20  $\mu$ l) and Total RNA from HL60 cells (100  $\mu$ g/lane) prepared as described in text and were electrophoresed on 15 % polyacrylamide gel (7.5 M urea, 1  $\times$  TBE). The rectangles surrounded by broken yellow lines represent portions to be excised.

a

clean razor blade for excision. Transfer the excised gel to a nuclease-free 1.5-ml tube. Grind the gel completely in the tube. Add 3 volumes of RNA Extraction Buffer. Elute the RNA from the gel by constant agitation at room temperature for 30 min. Centrifuge the tube at top speed (15,000 rpm) for 10 min using a microcentrifuge at 4°C to room temperature to precipitate gel pieces. Transfer the supernatant to a nuclease-free 1.5-ml tube. Add an equal volume of TE-saturated phenol to the supernatant. Vortex it for 15-30 sec. Centrifuge the tube at top speed for 5 min using a microcentrifuge at 4°C to room temperature. Transfer the supernatant to another nuclease-free 1.5-ml tube. Add an equal volume of chloroform to the supernatant. Vortex it briefly. Centrifuge the tube at top speed for 2 min at 4°C to room temperature. Transfer the supernatant to another nuclease-free 1.5-ml tube. Perform chloroform extraction as above again. Add 1/10 volume of 3 M NaOAc solution and 1/50 volume of 5  $\mu$ g/ml of glycogen. Mix it and centrifuge briefly to collect the contents at the bottom of the tube, then add three volumes of 100% ethanol to the solution. Put the tube at -80°C for 30 min or more, then centrifuge the tube at top speed for 10 min using a microcentrifuge at 4°C. To remove supernatant, centrifuge the tube again at top speed for 2 min at 4°C. Remove the supernatant completely and dry down the precipitate. Store it at -80°C until use.

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### Troubleshooting

Step	Problem	Probable Cause	Suggested Solution
I, II	Bands were bent on electrophoresis.	Overloading	Ten $\mu$ l or less than 10 $\mu$ l of sample can be applied to a well of 1.5 mm thickness and of 5 mm width for good separation.
		Different loading volumes	Load the ligated sample in a similar volume as Colored Guide Size Marker solution.
III	No bands were seen after electrophoresis of amplified products	Low amount of starting RNA	Use over 5 ng of small RNA. (over 100 $\mu$ g of total RNA)
		Procedural error and/or loss of sample during the cloning steps.	In this step, you can see band(s) around 60 bp. If not, confirm all steps of this protocol. For ethanol precipitations, use Glycogen and Sodium Acetate Solution in this kit, do not wash the precipitate with 70% ethanol. Confirm all steps till electrophoresis of amplified products. Control RNA in this kit will be useful to examine the cloning procedure. If you use 5 $\mu$ l of the Control RNA, you can see 3'-linker ligated product in the gel with ethidium bromide staining.
After III	Low percentage of inserted cDNA in T vectors	Low amount of starting RNA	Use over 5 ng of small RNA. (over 100 $\mu$ g of total RNA). Purification of PCR product, for example gel separation, may work. To avoid sequencing plasmids which contain only linker sequences, colony-PCR or PCR with mini-prep plasmids should be done prior to sequencing.
		Low content of small RNAs in starting RNA	Check the purification procedure of the starting RNA. Electrophoresis of the starting RNA is helpful to know the amount of tRNA, rRNA and other larger nucleic acids. Total RNA must be fractionated by polyacrylamide gel.

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