Cat. # RR065

For Research Use

TaKaRa

small RNA Cloning Kit

Product Manual

v201911Da

Table of Contents

I. Description

Many cellular RNAs do not encode proteins but do have important biological functions. These are classified as functional RNAs. Notably, small RNAs (18 to 26 bases) have received much attention. The function of these small RNAs include : [1, 2)]

(1) Suppression of gene expression at the mRNA content.

(2) Suppression of gene expression at the translational step.

Cloning and analyzing small RNA molecules is one method used to assess their expression patterns. Unlike mRNAs, small RNAs lack poly-A tails. Therefore, cloning small RNA molecules involves ligation of RNA or RNA/DNA chimeric adaptors to the 5′ and 3′ ends of purified small RNAs followed by reverse transcription and PCR.

This kit is intended for cDNA synthesis and amplification for cloning small RNA molecules. By using magnetic beads, this kit provides a simplified workflow that does not require gel extraction of nucleic acids after adaptor ligation. Therefore, small RNA-derived cDNAs can be amplified by a simple procedure. The amplified cDNA can then be used directly for TA cloning. Alternatively, cloning may be performed using the restriction enzyme site in the adaptor sequence.

Figure 1 (p. 4) shows an overview of the protocol for cloning small RNA using this kit. The small RNA may be prepared from total RNA by using electrophoresis and gel extraction.

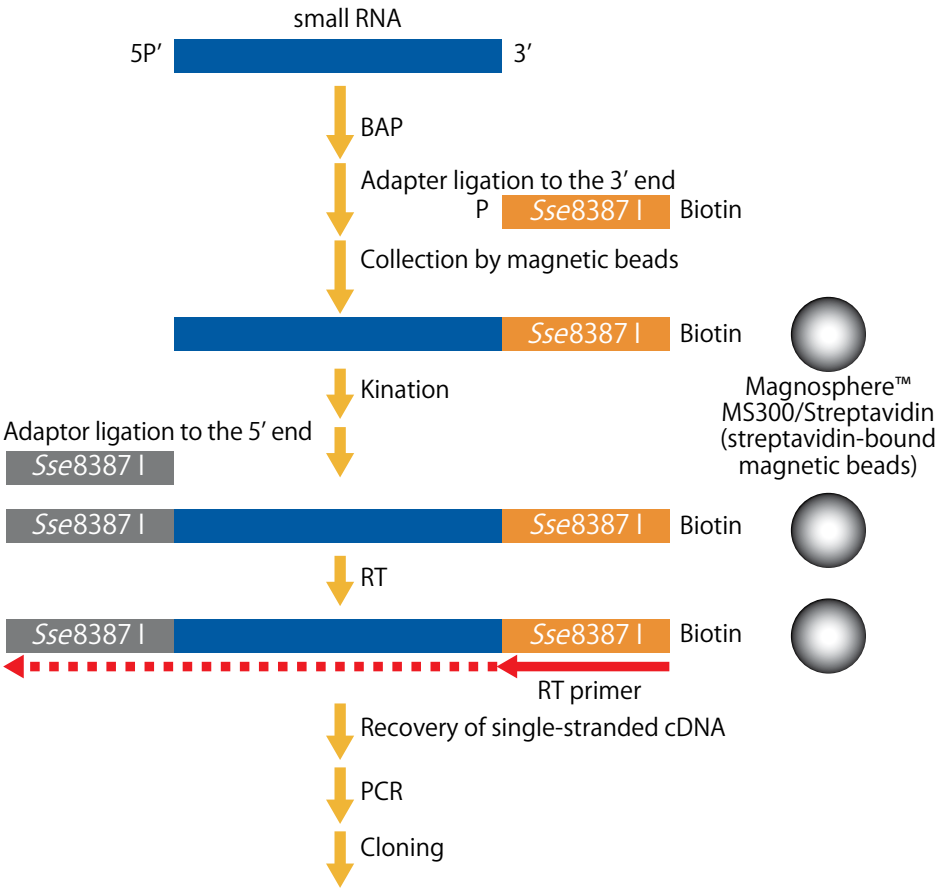

Figure 1. Overview of small RNA cloning protocol

1 Treat small RNA with BAP to dephosphorylate the 5′ ends.
2 Ligate the biotinylated RNA/DNA chimeric adaptors to the 3′ end of the BAP-treated RNA.
3 Collect the biotinylated adaptor ligated small RNA using streptavidin-bound magnetic beads and phosphorylate the 5′ end by kination.
4 After binding the RNA/DNA chimeric adaptors to the 5′ end of the molecules, perform a reverse transcription reaction with M-MLV reverse transcriptase [3)] using the RT primer.
5 Elute cDNA from the beads and perform PCR.
6 Clone the amplified PCR products.

II. Components (for 10 reactions)

Package 1 (store at -20℃) :

(1)	RNase Inhibitor (40 U/μl)	50 μl
(2)	10X BAP Buffer	200 μl
(3)	Alkaline Phosphatase (BAP) (0.4 U/μl)	20 μl
(4)	0.1% BSA	60 μl
(5)	3′ adaptor*1, 2	10 μl
(6)	T4 RNA Ligation Buffer	600 μl
(7)	T4 RNA Ligase (40 U/μl)	20 μl
(8)	5X T4 PNK Buffer	100 μl
(9)	T4 Polynucleotide Kinase (T4 PNK) (10 U/μl)	10 μl
(10)	5′ adaptor*1	10 μl
(11)	5X RT Buffer	40 μl
(12)	dNTP Mixture (2.5 mM each)	90 μl
(13)	PCR-R & RT-Primer (50 pmol/μl)*1, 3	20 μl
(14)	RTase (RNase H free) (200 U/μl)	10 μl
(15)	2X PCR Buffer	250 μl
(16)	PCR Primer F (50 pmol/μl)*1	10 μl
(17)	*TaKaRa Ex Taq*® HS (5 U/μl)	10 μl
(18)	Control RNA (5 pmol/μl)*4	10 μl

Package 2 (store at 4℃) :

(19)	3 M Sodium Acetate (pH5.2)	200 μl
(20)	Dr. GenTLE™ Precipitation Carrier*5	80 μl
(21)	2X B/W Buffer	2.7 ml
(22)	Magnosphere™ MS300/Streptavidin	100 μl
(23)	RNase-free dH_2O	10 ml

＊1 The 3′ adaptor, the 5′ adaptor, PCR Primer F, and PCR-R & RT-Primer include *Sse*8387 I restriction enzyme sites (see section VIII. Primer Sequences). By digesting with *Sse*8387 I, PCR products can be ligated to a vector cleaved by *Pst* I. (Note : This kit does not contain restriction enzymes, a cloning vector, etc. See section IV. Materials Required but not Provided.)

＊2 The 3′ adaptor is biotinylated.

＊3 This is a reverse primer for PCR. It can also be used as a reverse transcription primer.

＊4 The Control RNA is a synthetic 21-mer RNA with a 5′ phosphate (see section VIII. Primer Sequences).

＊5 This component is same as Dr. GenTLE Precipitation Carrier (Cat. #9094).

III. Storage

Package 1 : -20℃
Package 2 : 4℃

IV. Materials Required but not Provided

[Reagents]
Phenol / Chloroform / Isoamyl alcohol (25 : 24 : 1, v/v/v)
Chloroform / Isoamyl alcohol (24 : 1, v/v)
Ethanol
TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
0.1 N NaOH
DNA Size Marker
e.g., 20 bp DNA Ladder (Cat. #3409A/B)
RNA Size Marker
e.g., 14-30 ssRNA Ladder Marker (Cat. #3416)
10% polyacrylamide gel (non-denaturing)
15% polyacrylamide gel (denaturing)
Restriction enzyme *Sse*8387 I (Cat. #1183A/B)
Cloning vector
E. coli competent cells
Ligation kit
e.g., DNA Ligation Kit, Mighty Mix (Cat. #6023)
RNA purification reagent
e.g., RNAiso Plus (Cat. #9108/9109)*

[Equipment]
Microcentrifuge
Water bath or heat block (set at the temperatures below)
15℃, 25℃, 37℃, 42℃, 70℃
Micropipette, and Pipette Tips (with filters)
Microcentrifuge Tube
Thermal Cycler
0.2 ml tubes for PCR
Agarose gel electrophoresis equipment
Polyacrylamide gel electrophoresis equipment
Magnetic Stand
e.g., Magnetic Stand (6 tubes) (Cat. #5328)*

* Not available in all geographic locations. Check for availability in your area.

V. Precautions Before Use

1. Sterilization of Equipment

Although commercially available sterilized disposable plastic for lab use is usually RNase-free and may be used for experiments directly, microcentrifuge tubes, tips for micropipettes and other plasticware should be used only after autoclaved.

When using glassware, perform dry-heat sterilization at 160℃ for at least 2 hours. Equipment that cannot be dry-heat sterilized should be treated with 0.1% diethyl pyrocarbonate (DEPC) solution at 37℃ for 12 hours, followed by autoclaving before use (to prevent RNA carboxymethylation by DEPC). Designate equipment exclusively for RNA experiments and do not mix with apparatuses for other uses. In addition, since a major source of RNase contamination is sweat and saliva, wear gloves and a mask when conducting experiments.

2. Preparation of Reagents

Prepare reagent solutions with 0.1% DEPC-treated water as much as possible and autoclave before use. If reagents cannot be autoclaved, prepare the solution using previously sterilized equipment and water. Then perform filter sterilization on the prepared solution prior to use in the protocol. Use only solutions and distilled water designated exclusively for RNA experiments.

3. Preparation of RNA Samples

In order to clone small RNAs, protect the total RNA from degredation as much as possible. Prepare RNA from cells or tissues as soon as possible after harvesting the samples. If immediate RNA preparation is not possible, store samples at -80℃ or under liquid nitrogen until use.

(1) total RNA preparation

Use Cesium Chloride Density-Gradient Centrifugation, Acid Guanidinium Thiocyanate Phenol Chloroform (AGPC method), or commercially available kits and reagents for RNA extraction and purification. When using commercially available kits and reagents, confirm that they are capable of collecting small RNA molecules.

RNAiso Plus (Cat. #9108/9109)*, NucleoSpin miRNA (Cat. #740971.10) and NucleoSpin miRNA Plasma (Cat. #740981.10) are recommended.

(2) Purification of small RNA

For separation and purification of small RNA fractions, a denaturing polyacrylamide gel (e.g., 15% acrylamide: bisacrylamide (19 : 1)/ 7 M Urea/ 0.5X TBE gel) is commonly used.

We recommend using the 14-30 ssRNA Ladder Marker (Cat. #3416) to determine the target size of the RNA fraction.

For gel extraction of small RNAs, the "crush and soak" method can be used. When concentrating the RNA by ethanol precipitation, dissolve the RNA pellet in approximately 10 μl of RNase-free dH_2O. The use of Dr. GenTLE Precipitation Carrier (Cat. #9094) is recommended.

(3) RNA Purity

This kit requires approximately 1 - 100 ng (about 0.1 - 10 pmol) of small RNA per reaction. Although it is difficult to quantify purified small RNA accurately, instruments such as the Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA6000 Pico LabChip Kit (Agilent Technologies) allow accurate

estimation of quantities and size distribution of purified small RNA (see Figure 2). Alternatively, purified small RNA can be quantified by measuring the OD_{260} using a spectrophotometer.

* Not available in all geographic locations. Check for availability in your area.

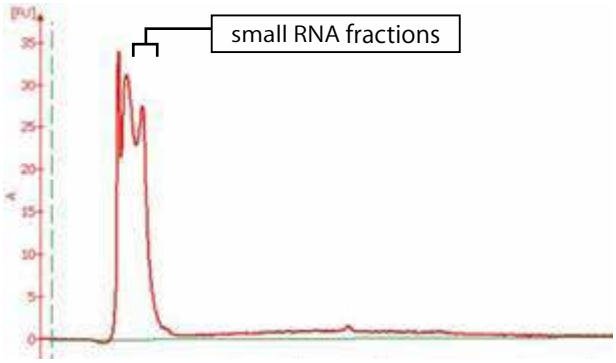

Figure 2. Example of small RNA analysis using the Agilent Bioanalyzer

VI. Protocol

VI-1. BAP Treatment of small RNA

1) Add [23] RNase-free dH_2O to 1 - 100 ng small RNA (dissolved in RNase-free dH_2O), to obtain a total volume of 42 μl.
2) Prepare the following reaction mixture in a microcentrifuge tube.

Reagent	Volume
small RNA	42 μl
[1] RNase Inhibitor	1 μl
[2] 10X BAP Buffer	5 μl
[3] BAP	2 μl
Total	50 μl

3) Incubate for 1 hour at 37℃ after mixing gently by pipetting or tapping.
4) Add 50 ul of [23] RNase-free dH_2O to obtain a total volume of 100 μl, then add an equivalent volume (100 μl) of phenol / chloroform / isoamyl alcohol and vortex for 5 - 10 sec.
5) Centrifuge at 4℃, 15,000 rpm for 5 min and transfer the upper aqueous layer to a new tube. Avoid transferring the interface.
6) Add an equivalent volume of phenol / chloroform / isoamyl alcohol and vortex for 5 - 10 sec. Then, centrifuge at 4℃,
15,000 rpm for 5 min and transfer the upper aqueous layer to a new tube.
7) Add an equivalent volume of chloroform / isoamyl alcohol and vortex for 5 - 10 sec.
8) Centrifuge at 4℃, 15,000 rpm for 5 min and transfer the upper aqueous layer to a new tube.
9) Add one-tenth volume of [19] 3 M Sodium Acetate, 4 μl of [20] Dr. GenTLE Precipitation Carrier, and 2.5 volumes of ethanol to the collected aqueous layer and mix well.
10) Let stand at -80℃ for 1 hour. Centrifuge at 4℃, 15,000 rpm for 1 hour and then remove the supernatant without disturbing the pellet.
11) Rinse the pellet with 80% ethanol.
12) Air-dry the pellet, then dissolve the pellet in 14 μl of [23] RNase-free dH_2O. To facilitate dissolving the pellet, do not dry excessively.

VI-2. 3' Adaptor Ligation

1) Prepare the following reaction mixture in a microcentrifuge tube.

Reagent	Volume
[4] 0.1% BSA	3 μl
[1] RNase Inhibitor	1 μl
[5] 3' adaptor	1 μl
BAP-treated small RNA	14 μl
Total	19 μl

2) Add 30 μl of [6] T4 RNA Ligation Buffer and mix thoroughly by pipetting. This buffer is viscous; pipette slowly and carefully.

3) Add 1 μl of [7] T4 RNA Ligase and mix well by pipetting.
4) Incubate at 15℃ for 1 hour.

VI-3. Adsorption to Magnetic Beads

1) Mix 210 μl of [21] 2X B/W Buffer and 210 μl of [23] RNase-free dH_2O in a microcentrifuge tube to prepare 1X B/W Buffer.
2) Suspend the [22] Magnosphere™ MS300/Streptavidin (magnetic beads) well, and dispense 10 μl aliquots into new microcentrifuge tubes.
3) Place the tubes in the magnetic stand, leave them for 30 sec, and then remove the supernatant from each tube.
4) Add 10 μl of [21] 2X B/W Buffer and vortex gently. Briefly centrifuge the tubes, place them in the magnetic stand, and leave them for 30 sec. (Note: bubbles may form upon vortexing because B/W Buffer contains Triton X-100. Formation of bubbles does not inhibit the reaction.)
5) Remove the supernatant, add 50 μl of [21] 2X B / W Buffer, and then mix.
6) Add 50 μl of the magnetic bead suspension prepared in VI-3-5 to the ligation reaction mixture prepared in step VI-2-4 and mix by pipetting.
7) Incubate at 25℃ for 1 hour.
8) Place tubes in the magnetic stand, leave them for 30 sec.
9) After removing the supernatant, add 100 μl of 1X B/W Buffer. Vortex briefly to wash the beads.
10) Place the tubes in the magnetic stand and leave them for 30 sec.
11) After removing the supernatant, add 100 μl of [23] RNase-free dH_2O and mix gently. (Do not remove the solution until ready to proceed with step VI-4-2 below.)

VI-4. 5′ Terminal Phosphorylation

1) Prepare the following reaction mixture in a microcentrifuge tube on ice.

Reagent		Volume
[23]	RNase-free dH_2O	38 μl
[1]	RNase Inhibitor	1 μl
[8]	5X T4 PNK Buffer	10 μl
[9]	T4 Polynucleotide Kinase	1 μl
Total		50 μl

2) Place the tubes from step VI-3-11 in the magnetic stand for 30 sec and remove the supernatant from the beads. Add 50 μl of the reaction mixture prepared in step VI-4-1 and suspend the beads gently by pipetting or tapping.
3) Incubate at 37℃ for 30 min.
4) Place tubes in the magnetic stand and leave them for 30 sec.
5) After discarding the supernatant, add 100 μl of 1X B/W Buffer. Vortex briefly to resuspend the beads.
6) Place tubes in the magnetic stand and leave them for 30 sec.
7) After removing the supernatant, add 100 μl of [23] RNase-free dH_2O and mix gently. (Do not remove the solution until ready to proceed with step VI-5-2 below.)

VI-5. 5′ Adaptor Ligation

1) Prepare the following reaction mixture in a microcentrifuge tube on ice and mix well.

Reagent	Volume
[23] RNase-free dH_2O	14 μl
[6] T4 RNA Ligation Buffer *	30 μl
[4] 0.1% BSA	3 μl
[10] 5′ adaptor	1 μl
[1] RNase Inhibitor	1 μl
[7] T4 RNA Ligase	1 μl
Total	50 μl

* Pipette this buffer slowly and carefully because it is viscous.

2) Place the tubes from step VI-4-7 in the magnetic stand for 30 sec. and remove the supernatant from the beads. Add the reaction mixture prepared in VI-5-1 and resuspend the beads by thoroughly by pipetting.
3) Incubate at 15℃ for 1 hour.
4) Add 50 μl of [23] RNase-free dH_2O and mix well. Place tubes in the magnetic stand and leave for 30 sec.
5) Remove the supernatant and add 100 μl of 1X B/W Buffer. Vortex briefly to resuspend the beads.
6) Place tubes in the magnetic stand and leave them for 30 sec.
7) After removing the supernatant, add 100 μl of [23] RNase-free dH_2O and mix gently. (Do not remove this solution until ready to proceed with step VI-6-2 below.)

VI-6. Reverse Transcription

1) Prepare the following reaction mixture in a microcentrifuge tube on ice.

Reagent	Volume
[11] 5X RT Buffer	4 μl
[12] dNTP Mixture	4 μl
[1] RNase Inhibitor	1 μl
[13] PCR-R & RT-Primer	1 μl
[14] RTase (RNase H free)	1 μl
Total	11 μl

2) Place the tubes from step VI-5-7 in the magnetic stand for 30 sec and remove the supernatant from the beads. Add 9 μl of [23] RNase-free dH_2O and incubate at 70℃ for 5 min, then place on ice for 1 - 2 min.
3) Add 11 μl of the reaction mixture prepared in VI-6-1, to obtain a total volume of 20 μl. Mix and incubate at 42℃ for 1 hour.
4) Place tubes in the magnetic stand and leave for 30 sec.
5) Remove the supernatant and add 100 μl of 1X B/W Buffer. Vortex briefly to resuspend the beads.
6) Place tubes in the magnetic stand and leave them for 30 sec.
7) After removing the supernatant, add 100 μl of [23] RNase-free H_2O and mix gently. (Do not remove this solution until ready to proceed with step VI-7-2 below.)

VI-7. PCR

1) Prepare the following reaction mixture in a microcentrifuge tube on ice.

Reagent	Volume
[23] RNase-free dH_2O	12 μl
[15] 2X PCR Buffer	25 μl
[12] dNTP Mixture	5 μl
[16] PCR Primer F	1 μl
[13] PCR-R & RT Primer	1 μl
[17] *TaKaRa Ex Taq*® HS	1 μl
Total	45 μl

2) Place the tubes from step VI-6-7 in the magnetic stand for 30 sec and remove the supernatant from the beads. Add 5 μl of 0.1 N NaOH and mix by tapping the tube. (Note: the solution should not be suspended by pipetting because beads will stick to the tip.)

3) Incubate at 25℃ for 5 min. Then, place tubes in the magnetic stand and leave them for 30 sec.

4) Remove the supernatant and transfer to a 0.2 ml PCR tube.

5) Add 45 μl of the reaction mixture prepared in VI-7-1 and mix well.

6) Set tubes in the thermal cycler and perform PCR under the following conditions.

94℃	2 min	
94℃	30 sec	┐
60℃	30 sec	├ 15 cycles
72℃	30 sec	┘
72℃	3 min	
4℃		

7) Analyze 5 μl of the reaction on a 10% non-denaturing polyacrylamide gel (See Figure 3).

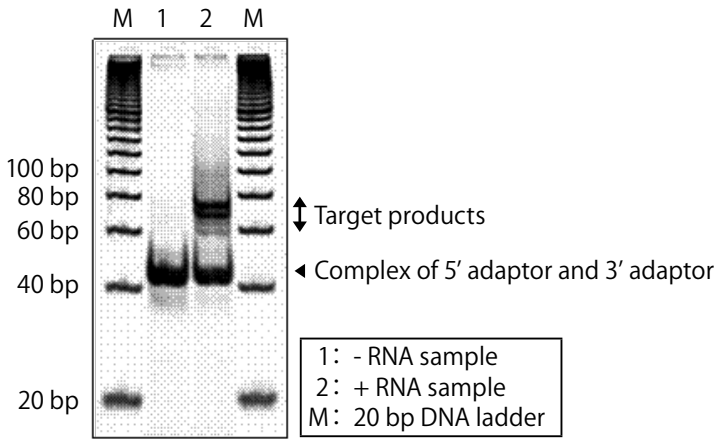

Figure 3. Result of reactions using 10 kinds of RNA samples (16 nt - 30 nt) (detected by the FMBIO II Multi-View).

One-tenth of the PCR reaction volume was subjected to electrophoresis on a non-denaturing 10% polyacrylamide gel and products were detected using the FMBIO II Multi-View and SYBR® Green I.

8) Add TE buffer to the remainder of the PCR products to obtain a final volume of 100 μl. Add an equal volume of phenol / chloroform / isoamyl alcohol and vortex well for 5 - 10 sec.
9) Centrifuge at 4℃, 15,000 rpm for 5 min and transfer the upper aqueous layer to a new tube. (Avoid transferring the interface.)
10) Add an equal volume of chloroform / isoamyl alcohol and vortex for 5 - 10 sec.
11) Centrifuge at 4℃, 15,000 rpm for 5 min and transfer the upper aqueous layer to a new tube.
12) Add one-tenth volume of [19] 3 M Sodium Acetate, 4 μl of [20] Dr. GenTLE Precipitation Carrier, and 2.5 volumes of ethanol to the aqueous layer and mix well.
13) Place at -80℃ for 30 min. Centrifuge at 4℃, 15,000 rpm for 15 min and then discard the supernatant without disturbing the pellet.
14) Wash with 80% ethanol.
15) Air-dry the pellet, then dissolve in 5 μl of TE. (Note: to facilitate dissolving the pellet, do not dry excessively.)
16) Proceed with Optional Protocols below for cloning of the PCR product.

Optional Protocols

Optional-1. TA Cloning of PCR Fragment

1) Add 1 μl of 6X Loading Buffer (36% glycerol, 30 mM EDTA, 0.05% BPB, 0.05% XC) to 5 μl of the PCR products prepared in VI-7 and perform electrophoresis using a non-denaturing 10% polyacrylamide gel. A 20 bp DNA ladder (Cat. #3409) is recommended as a size marker.
2) When the BPB dye front migrates approximately four-fifths of the gel length, stop electrophoresis. Stain the gel with freshly prepared staining solution (e.g., EtBr or SYBR Green I).
3) Using a clean razor blade, excise the portion of the gel corresponding to the fragment target size. (The target PCR product size is the sum of the RNAs used for the reaction and the length of PCR Primer F and PCR-R & RT-Primer. For example, when using [18] 21-mer Control RNA, the target product is 65 bp.)
4) Purify the DNA fragment from the excised gel by the "crush and soak" method or using a commercially available kits (e.g., NucleoSpin Gel and PCR Clean-up, Cat. #740609.10/.50/.250).
5) Mix 50 ng of T-Vector [e.g., T-Vector pMD20 (Cat. #3270), T-Vector pMD19(Simple) (Cat. #3271)] with the purified PCR fragments. Add an equivalent volume of Ligation Mix from the DNA Ligation Kit, Mighty Mix (Cat. #6023) and incubate at 16℃ for 30 min.
6) Transform chemically competent cells [e.g., E. coli JM109 Competent Cells (Cat.#9052)] using a portion of the ligation reaction. (When transformingelectro-competent cells, we recommend first performing ethanolprecipitation of the ligation reaction, followed by dissolving the DNA pellet in sterile purified water.)
7) Spread a suitable volume of cells on selective medium containing theappropriate antibiotic and incubate at 37℃.

Optional-2. Restriction digestion and cloning

1) Prepare the following reaction mixture in a microcentrifuge tube.

Reagent	Volume
Sterile purified water	34 μl
10X M Buffer	5 μl
0.1% BSA	5 μl
PCR fragment prepared in VI-7	5 μl
*Sse*8387 I (10 U/μl)	1 μl
Total	50 μl

2) Incubate at 37℃ overnight.

3) Add 5 μl of 3 M NaOAc (pH 5.2) and 125 μl of 100% ethanol. Let stand at -80℃ for 30 min. Centrifuge at 4℃, 15,000 rpm for 15 min.

4) Remove the supernatant without disturbing the pellet. Wash the pellet with 80% ethanol.

5) After air-drying, dissolve the pellet with 5 μl of TE buffer.

6) Add 1 μl of 6X Loading Buffer and perform electrophoresis on a non-denaturing 15% polyacrylamide gel. A 20 bp DNA ladder (Cat. #3409) is recommended as a size marker.

7) When the BPB dye front migrates to approximately four-fifths of the gel length, stop electrophoresis. Stain the gel with a freshly prepared stain solution (e.g., EtBr, SYBR Green I).

8) Using a clean razor blade, excise the portion of the gel corresponding to the fragment target size. (The PCR product size is the sum of the RNAs used for the reaction and the length of PCR Primer F and PCR-R & RT-Primer. When digesting with *Sse*8387 I, a fragment that is shortened by 22 bp is the target fragment. For example, when using the 21-mer Control RNA, the target product is 65 bp. After digestion with *Sse*8387 I, the target product is 43 bp.)

9) Purify the DNA fragment from the excised gel by the "crush and soak" methodor using a commercially available kits (e.g., NucleoSpin Gel and PCR Clean-up, Cat. #740609.10/.50/.250).

10) For cloning the *Sse*8387 I digest, use 50 ng of *Pst* I-digested vectors [e.g., pUC118 *Pst* I/ BAP (Cat. #3323)]. Add an equivalent volume of Ligation Mix from the DNA Ligation Kit, Mighty Mix (Cat. #6023) and incubate at 16℃ for 30 min.

11) Transform chemically competent cells [e.g., *E. coli* JM109 Competent Cells (Cat. #9052)] using a portion of the ligation reaction. (When transforming electro-competent cells, we recommend first performing ethanol precipitation of the ligation reaction, followed by dissolving the DNA pellet in sterile purified water.)

12) Spread a suitable volume of cells on selective medium containing the appropriate antibiotic and incubate at 37℃.

VII. Troubleshooting

If the expected results are not obtained, consider the following factors.

1. Control Experiment

   This kit includes a synthetic 21-mer RNA with a phosphorylated 5′ terminus as a control RNA. Perform the reaction using this control and confirm that the correct PCR product is obtained (Figure 4. Lanes 1, 2). If two bands appear as shown in Lanes 3 and 4, the BAP treatment of the RNA may be incomplete. Perform the BAP treatment again. If the correct target products are obtained from the Control RNA, try to increase the amount of the small RNA used for the reaction.

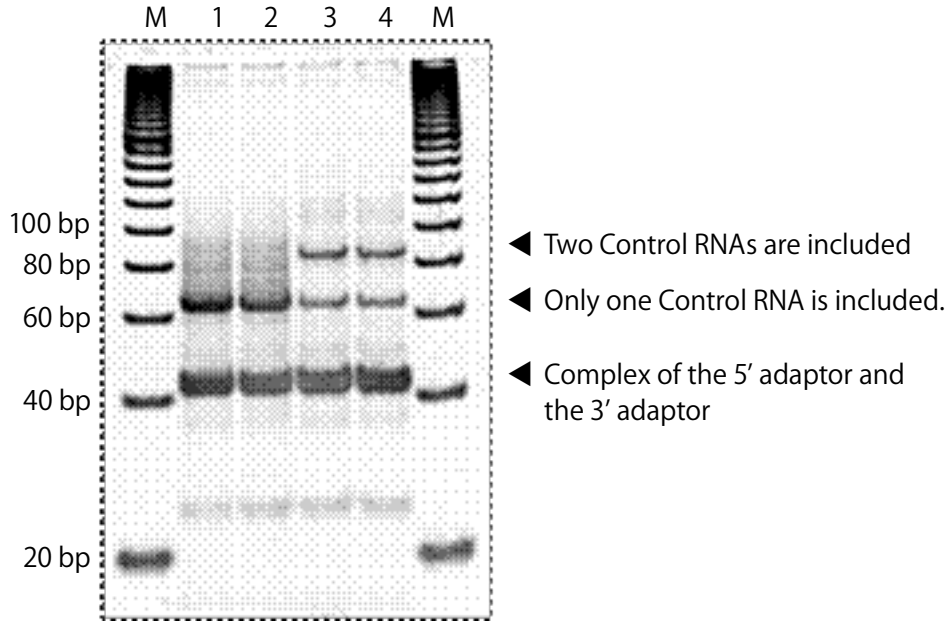

Figure 4. Gel electrophoresis of PCR products when using Control RNA (Detected by FMBIO II Multi-view)

Lane 1, 2 : BAP-treated Control RNA
Lane 3, 4 : BAP-untreated Control RNA. If the 5′ end of RNA is phosphorylated, PCR products with combined RNAs will appear.
M : 20 bp DNA Ladder

2. RNase contamination

   It is essential to prevent RNase contamination. Perform dry-heat sterilization or autoclave equipment and reagents used for the protocol as much as possible. Wear gloves when performing experiments.

3. T4 RNA Ligation Buffer

   The T4 RNA Ligation Buffer in this kit includes PEG to increase reaction efficiency. When pipetting this buffer, the point of the tip must be kept submerged in the solution until all of the solution volume is fully drawn into the tip. When the tip is removed from the solution, ensure that air does not enter the tip. When the buffer is added to the reaction mixture, take care to avoid introducing bubbles. To fully mix this solution, pipetting the solution about 10 times is required.

   Note :
   For T4 RNA Ligase, different ligation efficiency may occur depending on the terminal nucleotide type. Furthermore, 1 or more nucleotides may be removed from the RNA termini during the ligation reaction.

4. Gel extraction after PCR

   If the amount of DNA applied to a well is large or electrophoresis is carried out rapidly, the DNA bands may smear at the well edges as seen in Figure 3. In this case, reduce the amount of DNA loaded in the well, or use a larger well. Alternatively, results may be improved by performing electrophoresis slowly. In addition, be careful not to include the lower band (complex of adaptors) in gel slice. When the edges of the band are smeared, we recommend omitting both ends when excising the gel slice and performing gel extraction.

5. Other

   When handling multiple samples at the same time, pay close attention to the removal and addition of solutions when using the magnetic beads. Too much time between solution removal and addition of the next solution may allow the magnetic beads to dry and the reaction may not work properly. It is recommended that the next solution be added just after removing the previous solution.

VIII. Primer Sequences

The sequence of the primers is as follows:

- PCR-R & RT-Primer
  5′ -GTCTCTAG**CCTGCAGG**ATCGATG-3′
  This primer is used for the reverse transcription and as a reverse primer for PCR. It includes the *Sse*8387 I recognition sequence (shown in bold).

- PCR-Primer F
  5′ -AAAGAT**CCTGCAGG**TGCGTCA-3′
  This primer includes the *Sse*8387 I recognition sequence (shown in bold).

- Control RNA
  5′ p-AGAUGACGGCAACUACAAGAC-3′

IX. References

1) Bartel D P. *Cell.* (2004)**116**:281.
2) Aravin A, *et al. FEBS Lett.* (2005)**579**:5830.
3) Roth M J, *et al. J Biol Chem.* (1985)**260**: 9326.

X. Related Products

RNAiso Plus (Cat. #9108/9109)
NucleoSpin miRNA (Cat. #740971.10/.50/.250)
NucleoSpin miRNA Plasma (Cat. #740981.10/.50/.250)
NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250)
14-30 ssRNA Ladder Marker (Cat. #3416)
Dr. GenTLE™ Precipitation Carrier (Cat. #9094)
Alkaline Phosphatase (*E. coli* C75) (Cat. #2120A/B)
T4 RNA Ligase (Cat. #2050A/B)
Magnetic Stand (6 tubes) (Cat. #5328)*
T4 Polynucleotide Kinase (Cat. #2021/A/B/S)
PrimeScript™ Reverse Transcriptase (Cat. #2680A/B)
TaKaRa Ex Taq® Hot Start Version (Cat. #RR006A/B)
dNTP Mixture (2.5 mM each) (Cat. #4030)
20 bp DNA Ladder (Cat. #3409A/B)
DNA Ligation Kit, Mighty Mix (Cat. #6023)
*Sse*8387 I (Cat. #1183A/B)
T-Vector pMD20 (Cat. #3270)
T-Vector pMD19 (Simple) (Cat. #3271)
pUC118 *Pst* I/BAP (Cat. #3323)
E. coli JM109 Competent Cells (Cat. #9052)

* Not available in all geographic locations. Check for availability in your area.