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Original Article

Amplification of unknown RNAs and RNA mixtures based on unique restriction enzyme cleavage *in vitro*

Fangyi Yang[1†], Jie Wang[2†], Yajun Ji[3†], Hao Cheng[1], Junting Wan[1], Zhiyu Xiao[2], and Guochun Zhou[1*]

[1]Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China
[2]Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou 510120, China
[3]Department of Oncology, The First People's Hospital of Lianyungang, Lianyungang 222002, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-13433968219; Fax: +86-20-32015351; E-mail: allspringzhou@yahoo.com

Small RNAs, generally expressed at low levels, are difficult to reach usable levels from limited material. In this study, we have developed a novel method to amplify target RNA. The amplification procedure was carried out by sequential RT-PCR, effective separation, restriction enzymatic cleavage of cDNA strand, and run-off transcription *in vitro* of target RNA from its cDNA. Introduction of a unique stem-loop linker into cDNA strand is the key step to form a unique restriction enzyme recognition sequence that is not in cDNA sequence of target RNA. This method can be used to amplify RNA samples from various origins and has many advantages in amplifying unknown small RNAs and small RNA mixtures. The amplified RNA has the full sequence of original RNA except for an extra 5′ G and an additional 3′ A or C. The method worked well for amplifications of a microRNA, a piwi interacting RNA and two small RNA mixtures.

Keywords RNA amplification; stem-loop linker; unknown RNA; RNA mixture

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Introduction

In recent years, many reports have demonstrated that epigenetic regulation by small RNAs happens in a wide range of eukaryotic and prokaryotic organisms [1], which attracts a huge surge of interest in the biology and technology of small RNAs. At least four naturally occurring and highly conserved small RNAs (18–35 nt in length), microRNAs (miRNAs) [2,3], repeat-associated siRNAs (rasiRNAs) [4], piwi interacting RNAs (piRNA) [5–9], and small nucleolar RNAs (snoRNAs) [10] are found to play significant roles in cell development, differentiation, and communication [11,12]. Small RNAs regulate gene expression by catalyzing the cleavage of messenger RNA (mRNA) [12–14], by repressing mRNA translation [14] or by interacting with specific protein(s) [5–9,15].

The expression levels of small RNAs vary greatly among species and tissues. The low available quantity of small RNA is a common obstacle in many studies, especially for some tissue-specific small RNAs from rare sources that are difficult to obtain. On the other hand, limited RNA source, which is likely to contain up to a hundred different small RNAs at most cases, can preclude the enrichment of all discrete small RNAs to usable levels for subsequent experiments. Some methods [16] have been established to globally amplify RNA, but these amplified RNAs were added by one or two appendages (generally longer than 5 nt at one terminus). The appendage sequence might not be a problem for the long RNA, but it may affect the functions of the small RNA. There are also some established methods to detect and quantify known small RNAs [17–19] or to express known miRNAs by constructing an expression vector [20]. However, these methods do not fit well for exact amplification and expression of unknown small RNAs. Unlike mRNA with a poly(A) template or a known small RNA that is feasible for primer design, the unknown small RNA or the small RNA mixture lacks a known or common marker for its precise amplification and expression. To faithfully enrich small RNAs from limited samples, a reliable and reproducible method is required to precisely amplify small RNA mixtures or individual unknown small RNAs before their sequences are determined. We present here a novel and practical RNA amplification method that amplifies unknown small RNAs precisely and without bias.

Our protocol requires specific acceptor, adaptor, and a special stem-loop linker. First, the unknown small RNA or the small RNA mixture is amplified by RT-PCR after attachments of the adaptor and the acceptor. The cDNA

strand/s of the target RNA/s is/are then separated from the PCR product. Then, the introduction of the unique stem-loop linker makes one restriction endonuclease (RE) cleavage site at the 5′-end of the cDNA. After digestion of this site, *in vitro* run-off transcription generates amplicons of the target RNA. In this paper, this method was used to successfully amplify miR122 (miRNA, 23 nt), hsa_piR_021065 (piRNA, 30 nt) [21], and two RNA mixtures of bp-s1 (~33 nt) and bp-s2 (~40 nt) that were obtained from tumor-adjacent tissue of a liver cancer patient.

Materials and methods

Materials

T4 RNA ligase, T4 DNA ligase, T4 polynucleotide kinase, *Nla*III, and Nt.BstNBI were purchased from New England BioLabs (Beijing) Ltd. (Beijing, China). Calf intestine alkaline phosphatase (CIAP), reverse transcriptase M-MLV, Pyrobest DNA polymerase, Klenow fragment, T7 RNA polymerase, DNase I, and small RNA gel extraction kit were obtained from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). NucleoSpin® Extraction II was from Germany and International Macherey-Nagel GmbH & Co. (Dueren, Germany). Dynabeads M280-Streptavidin was from Invitrogen (Carlsbad, USA). The mirVana miRNA isolation kit (Ambion) was purchased from Applied Biosystems Inc. (Foster City, USA).

Oligonucleotides of DNA and RNA used in this study were synthesized by ABI 3900 synthesizer in Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China) and by ABI 394 RNA Synthesizer in TaKaRa Biotechnology (Dalian) Co., Ltd., respectively.

Tumor-adjacent liver tissue was collected from a male liver cancer patient who underwent curative surgery for liver cancer at Department of Hepatobiliary Surgery, Sun Yat-sen Memorial Hospital of Sun Yat-sen University (Guangzhou, China) in 2007. The patient was informed that tests in this study were designed for research only.

All the reactions were carried out according to or with modification of the protocols recommended by the reagents' manufacturers. Analyses of these reaction products were done by 15% denaturing (with 8 M urea) or 8% native (without urea) polyacrylamide (acryl/bis-acryl = 19:1) gel electrophoresis (PAGE). Gels were stained with SYBR Green II or ethidium bromide. DNA or RNA was extracted from target band gel slices using a small RNA gel extraction kit (TaKaRa) and eluted with RNase-free H_2O.

Ligation of miR122 with the adaptor SL1

The ligation reaction of miR122 with SL1 was carried out in a 50 μl reaction mixture containing 20 μl SL1 (50 μM), 5 μl miR122 (50 μM), 5 μl 10× T4 RNA ligase buffer, 5 μl T4 RNA ligase (20 U/μl), 1 μl RNase inhibitor (40 U/μl), 14 μl RNase-free H_2O. The reaction mixture was incubated at 37°C for 24 h and then fractionated by 15% denaturing PAGE. After the gel was stained, the expected band (42 nt) was cut out and extracted using a small RNA gel extraction kit to give purified P1.

Phosphorylation

The phosphorylation reaction was carried out to generate P2 in a 15 μl reaction mixture containing 6 μl purified P1 (~400 ng from the above step), 1.5 μl 10× T4 RNA ligase buffer, 1 μl T4 polynucleotide kinase (10 U/μl) and 6.5 μl RNase-free H_2O at 37°C for 2 h. The reaction mixture was heated at 65°C for 20 min to inactivate T4 polynucleotide kinase.

Ligation of P2 with acceptor LL1

The ligation of P2 with LL1 was done in a reaction mixture containing 15 μl P2 (from the above step), 1 μl LL1 (100 μM), 3.0 μl 10× T4 RNA ligase buffer, 2 μl T4 RNA ligase (20 U/μl), 0.5 μl RNase inhibitor (40 U/μl), 8.5 μl RNase-free H_2O. The reaction mixture was incubated at 37°C for 24 h and then fractionated by 15% denaturing PAGE. After the gel was stained, the expected band (72 nt) was cut out and extracted using a small RNA gel extraction kit to give purified P3.

Reverse transcription

To generate a small RNA cDNA, 20 μl purified P3 (~100 ng) and 1 μl forward primer SLP1 (10 μM) were mixed. After the mixture was heated for 5 min at 94°C and then cooled to 16°C, 8 μl 5× M-MLV buffer, 4 μl dNTP mixture (2.5 mM each dNTP), 0.25 μl RNase inhibitor (40 U/μl), 1 μl reverse transcriptase M-MLV (200 U/μl), and 6 μl RNase-free H_2O were added into the reaction mixture. The mixture was incubated at 42°C for 1 h and the reverse transcriptase was inactivated by incubation at 70°C for 15 min.

PCR

PCR was performed using 5 μl 10× pyrobest buffer, 2 μl RT product P4 (small RNA cDNA), 3 μl dNTP mixture (2.5 mM each dNTP), 5 μl forward primer SLP1 (1 μM), 5 μl reverse primer LLP1 (1 μM), 30 μl RNase-free H_2O and 0.25 μl pyrobest DNA polymerase (5 U/μl) in a 50 μl reaction volume. A two-step PCR method (5 min at 98°C, then 30 cycles of 1 min at 98°C, and 1 min at 65°C) was used. After PCR, 10 μl of the PCR product was analyzed by 8% native PAGE. The non-incorporated primers were removed from PCR reactions using NucleoSpin Extraction II kit.

Immunomagnetic separation

Dynabeads M280-Streptavidin of 20 μl (10 mg/ml) was pre-washed three times in 2× B&W buffer (10 mM Tris–HCl, pH 7.5; 1 mM EDTA; 2.0 M NaCl) and then resuspended in 50 μl 2× B&W buffer. Fifty microliters (~1 μg) of the PCR product P5, in which one strand was 5′-biotinylated, was added to the suspension and then the mixture was incubated for 20 min at room temperature while keeping the beads suspended. After the unbound DNA was removed by washing three times with 50 μl 1× B&W buffer, 8 μl 0.1 M NaOH was added to the beads and the mixture was incubated for 10 min at room temperature with occasional mixing. The supernatant was obtained and neutralized with an equal volume of 0.1 M HCl. After the supernatant was fractionated by 8% native PAGE, the expected single-stranded P7 (71 nt) was cut out and extracted using a small RNA gel extraction kit to provide purified P7.

Ligation of nicked P8

5′-Phosphorylated P7 could be formed by using 5′-phosphorylated SLP1 primer in PCR or free P7 was phosphorylated by T4 polynucleotide kinase as usual. Formation of nicked P8 (annealed P7 and SL2) was conducted as following: 1 μl SL2 (100 μM) and 45 μl phosphorylated P7 were heated at 98°C for 5 min to denature and then cooled to 16°C to anneal. After 3 μl T4 DNA ligase (400U/μl) was added, the reaction mixture was incubated at 16°C for 19 h. The entire reaction product was run on 15% denaturing PAGE and stained.

Nt.BstNBI digestion of annealed products of Sde and SL2

One microliter of 10 μM Sde, 2 μl SL2 (10 μM), 2 μl Nt.BstNBI buffer 3, and 15 μl RNase-free H_2O were mixed. The mixture was heated at 98°C for 5 min to denature and then cooled to 16°C to anneal. After 1 μl Nt.BstNBI (10 U/μl) was added, the reaction mixture was incubated at 55°C for 4 h. The entire reaction product was analyzed on 15% denaturing PAGE and stained.

Digestion by Nt.BstNBI

Forty microliters (~1 μg) of the purified single-strand P7 (71 nt), 5 μl stem-loop linker SL2 (10 μM), and 5 μl Nt.BstNBI buffer 3 were mixed. Annealing was carried out by heating for 5 min at 98°C and then cooling to 16°C. Ligation of nicked P8 was conducted by T4 DNA ligase if ligated P8 was used for digestion; otherwise, nicked P8 directly proceeded to digestion. One microliter Nt.BstNBI (10 U/μl) was added to nicked P8 or ligated P8, and the reaction mixture was incubated at 55°C for 8 h. The entire reaction product was analyzed by 15% denaturing PAGE and the expected band (53 nt) was cut out and extracted using a small RNA gel extraction kit to give P9.

Filling-in of 5′-overhangs to form blunt ends

Forty microliters (~2 μg) of the cleavage substrate P9 and 1.5 μl T7 promoter (T7P, 100 μM) were mixed. After annealing (heating at 98°C for 5 min and then cooling to16°C), 5 μL of 10× Klenow fragment buffer, 1 μl dNTP mixture (2.5 mM each dNTP), and 4 μl Klenow fragment (2.5 U/μl) were added to the mixture. After incubation at 37°C for 3 h, the reaction mixture was analyzed by 8% native PAGE. The expected band (53 bp) was cut out and was extracted using a small RNA gel extraction kit to give blunt end dsDNA P11.

Run-off transcription *in vitro*

The transcription reaction was carried out in a 20 μl reaction containing 2 μl of 10× T7 RNA polymerase buffer, 2 μl DTT (50 mM), 4 μl NTP mixture (2.5mM each NTP), 0.5 μl RNase inhibitor (40 U/μl), 1 μl T7 RNA polymerase (10−50 U/μl), 10.5 μl (~500 ng) blunt end DNA P11. The reaction was incubated at 37°C for 2 h and treated with or without 4 μl DNase I (5 U/μl) or 2 μl RNase A (10 mg/ml) for 1 h. The reaction mixture was analyzed by 15% denaturing PAGE. The expected RNA bands (24~25 nt) were cut out and extracted using a small RNA gel extraction kit to provide purified amplicons of miR122.

In vitro amplification of piRNA

Amplification *in vitro* of hsa_piR_021065 (30 nt) [21] of a piRNA was carried out as same as the amplifying procedure of miR122. In order to reduce self-pairing of T7P with 18 bases of T7P (data not shown), T7PS (15 nt) with three bases of T7 promoter substituted T7P (30 nt) as transcription promoter in amplification of piRNA and later RNA mixtures of bp-s1 and bp-s2. T7P will be generated by Klenow fragment using T7PS as a filling-in primer.

In vitro amplification of bp-s1 and bp-s2

Small RNAs were isolated from a tumor-adjacent tissue of a male liver cancer patient using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. Concentration and purity of RNA were determined by UV spectrometry. Isolated small RNA mixtures were analyzed on 15% denaturing PAGE and the RNA bands of ~33 nt bp-s1 and ~40 nt bp-s2 were cut out and extracted by small RNA gel extraction kit. The bp-s1 or bp-s2 was treated with 40–120 U CIAP at 37°C for 2 h and dephosphorylated bp-s1 or bp-s2 was purified using 15% denaturing PAGE gel. By the same procedure as miR122 amplification, bp-s1 or bp-s2 was amplified

in vitro. Here, T7PS (15 nt) substituted T7P (30 nt) as T7P guide.

Results

General designs

To demonstrate our strategy, miR122 (5′-UGGAGUGUGA CAAUGGUGUUUGU-3′) was used as a target RNA. Nt.BstNBI and *Nla*III were chosen as example REs. The nicking endonuclease Nt.BstNBI (5′-GAGTCNNNN▼ N-3′/5′-NNNNNGACTC-3′) catalyzed a single-strand breaking 4 bases beyond the 3′-side of the recognition sequence (5′-GAGTC-3′/5′-GACTC-3′) and *Nla*III catalyzed double-strand palindromic cleavage at the 3′ of the recognition sequence (5′-CATG▼-3′/5′-CATG▲-3′). Based on the characteristics of Nt.BstNBI and *Nla*III, we constructed a chimera cleavage site (5′-GAGTCCATG▼-3′) recognized by both enzymes (**Fig. 1**, **Table 1**). The outline of the protocol is shown in **Fig. 1**.

An adaptor (SL1) was designed with the recognition sequence and cutting site(s) of REs Nt.BstNBI and *Nla*III. An acceptor (LL1) contained a T7 transcription promoter-related sequence that is recognized by T7 RNA polymerase. RT and PCR primers (SLP1 and LLP1) were based on SL1 and LL1, respectively. The sequence of SL1 was also the basis of the key stem-loop linker SL2 (**Table 1**), which guided and determined the formation of the exclusive restriction site(s). Sde was a single-strand DNA that contains two recognition sequences and 5′-termini could pair with SL2 precisely.

The adaptor, acceptor, primers, stem-loop linker and the promoter used were presented in **Table 1** and the results of gel electrophoresis were summarized in **Fig. 2**.

Introduction of an adaptor and an acceptor for target RNAs

The adaptor SL1 (**Table 1**) with recognition sequences for Nt.BstNBI and *Nla*III was introduced into the 3′-termini of the target RNAs and the acceptor LL1 (**Table 1**), containing the RNA sequence of the T7P, was ligated to the 5′-termini of the target RNAs. The adaptor SL1 was 5′-phosphorylated and should be 2′,3′-dideoxy or 3′ blocked at the 3′-terminus to prevent self-circularization during the ligation. In our study, the adaptor SL1 with 2′,3′-dideoxy form at the 3′-terminus and the acceptor LL1 with free -OH at both termini were used.

It is necessary to pretreat natural RNA samples with CIAP to remove the 5′-phosphate before ligation with the adaptor SL1. Ligation of SL1 with miR122 by T4 RNA ligase produced P1 [**Figs. 1** and **2(a)**]. P1 was phosphorylated by T4 polynucleotide kinase to produce P2. 5′-phosphorylated P2 was then ligated to the acceptor LL1 by T4 RNA ligase to yield the product P3 [**Figs. 1** and **2(a)**]. P3 (**Fig. 1**) contained recognition sequences for Nt.BstNBI and *Nla*III and the RNA sequence of the T7P. The efficiency of these two ligation steps of single-stranded RNA or/and DNA by T4 RNA ligase was crucial for the amplification of the RNA mixture when the original RNA sample was limited.

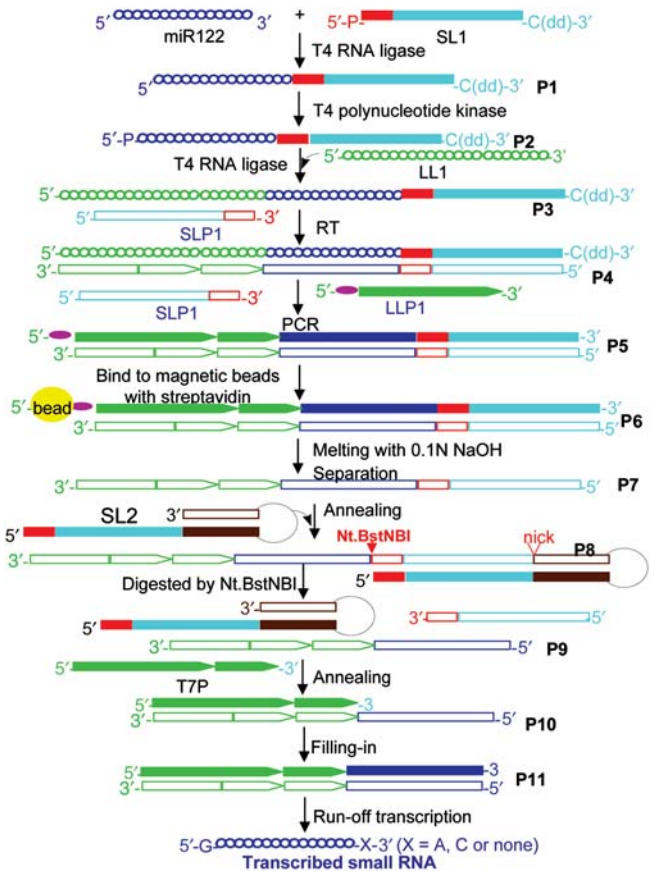

Figure 1 Flow diagram for RNA amplification Sequences depicted by solid and hollow shapes are complementary. P, phosphorylated at the terminus; C(dd), 2′,3′-dideoxy C; wavy line, RNA sequence; cyan, DNA sequence connected with recognition sequence of *Nla*III and Nt.BstNBI; red, DNA sequence between Nt.BstNBI recognition beginning site to cutting site (**Table 1**); blue, DNA or RNA sequence of target RNA; green, DNA or RNA sequence containing T7P-related sequence (**Table 1**); wedge shape, DNA sequence related to T7P (**Table 1**); pink, biotin moiety; yellow, magnetic bead with streptavidin; purple, stem-loop DNA sequence in SL2.

RT and PCR

Reverse transcription (RT) of P3 was conducted using SLP1 as RT primer. RT can be followed by PCR using SLP1 and LLP1 as PCR primers according to general RT-PCR procedure. SLP1 was designed as the complementary sequence of SL1 and LLP1 was 5′-DNA sequence (18 nt in length) of LL1 RNA. LLP1 was 5′-biotinylated and SLP1 was 5′-phosphorylated or without –OH at 5′-terminus. 5′-biotinylated primer LLP1 was designed for the convenience for solid phase immunomagnetic

Table 1 Sequences used for the amplification of miR122

Serial name	Sequence	Symbol[a]
miR122 RNA (23 nt)	5′-UGGAGUGUGACAAUGGUGUUUGU-3′	
Recognition sequence (9 bps)	5′-*CATG*GACTC-3′ 3′-*GTAC*CTGAG-5′	
SL1 DNA (19 nt)	5′-P-*CATG*GACTC(dN)$_m$C(dd)-3′	
LL1 RNA (30 nt)	5′-(N)$_n$UAAUACGACUCACUAUAG-3′	
SLP1 DNA (18 nt)	5′-(dNc)$_m$GAGTC*CATG*-3′	
LLP1 DNA (18 nt)	5′-Biotinyl-(dN)$_n$TAATAC-3′	
SL2 DNA (39 nt)	5′-*CATG*GACTC(dN)$_m$-stem-loop-3′	
T7P DNA (30 nt)	5′-(dN)$_n$TAATACGACTCACTATAG-3′	
T7PS DNA (15 nt)	5′-(dN)$_n$TAA-3′	
Sde DNA (70 nt)	5′-ATCTGTGCT*GAGTCCATG*ACAAACACCATTGTCACACTC*GAGTCCATG*AGTCGTATTAGATCGTTCACGA-3′	
hsa_piR_021065 (30 nt)	5′-UAAAUGUAAUCCAGCAUAUAAACAGAGCCA-3′	
bp-s1 (∼33 nt) and bp-s2 (∼40 nt)	Small RNA mixtures from tumor-adjacent tissue of a male liver cancer patient	

P, phosphorylated at 5′-terminus; C(dd), 2′,3′-dideoxy C; Biotinyl, biotinylation at 5′-terminus; italics, common recognition sequence and cutting site at the 3′ side of the recognition sequence (5′-CATG▼-3′) of *Nla*III and Nt.BstNBI; (N)$_n$, RNA sequence (n nt); (dN)$_n$, DNA sequence (n nt); (dN)$_m$ and (dNc)$_m$, complementary DNA sequence (m nt); underlined sequence: part- or full-length T7P sequence.

[a]The length of the symbol does not represent the exact length of RNA or DNA sequence.

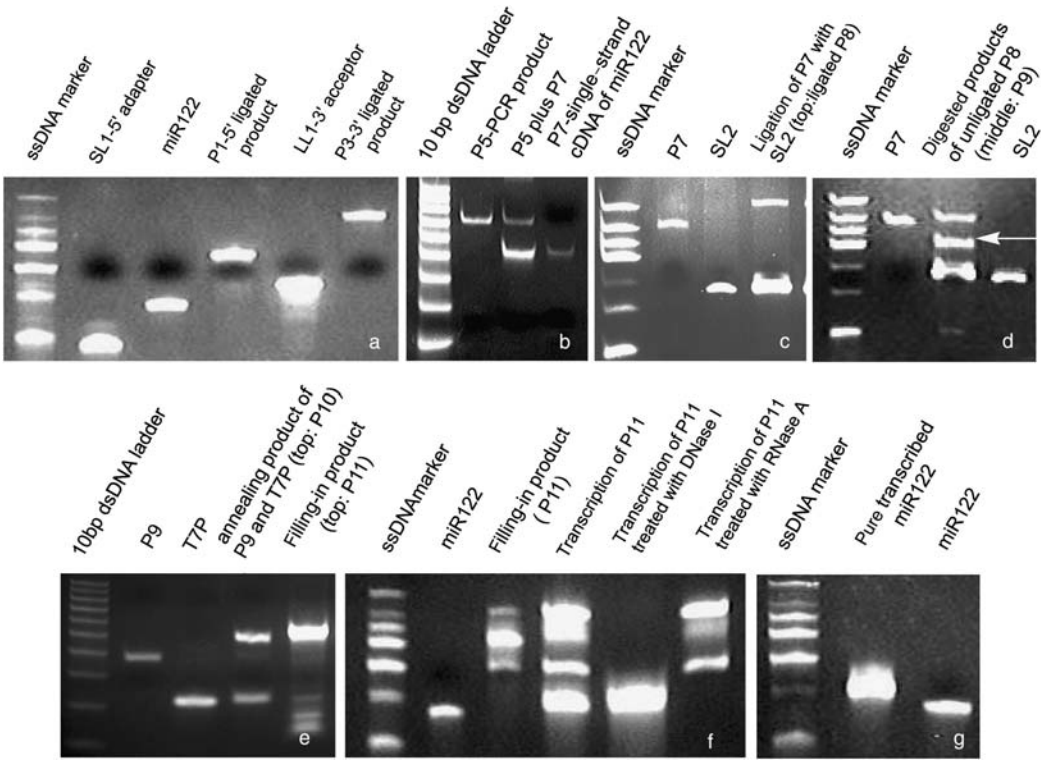

Figure 2 Gel illustrations of miR122 transcription (a) 15%, 8 M urea PAGE; (b) 8%, native PAGE; (c) 15%, 8 M urea PAGE; (d) 15%, 8 M urea PAGE; (e) 8%, native PAGE; (f) 15%, 8 M urea PAGE; (g) 15%, 8 M urea PAGE. ssDNA marker: from the up 96, 70, 60, 50, 40, 30 and 20 nt; 10 bp dsDNA ladder: from the up 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10 bp.

separation of two DNA strands after PCR amplification and 5′-phosphorylated SLP1 was used to enable ligation to nicked P8 at a later step.

After PCR amplification [**Fig. 2(b)**], we have an infinite source of the target RNA(s). If the original RNA sample is a mixture, a DNA pool that represents hundreds of different RNAs exists in proportion to the original RNA sample would be produced.

Immunomagnetic separation of cDNA strand and annealing with stem-loop linker

Biotin was introduced into one DNA strand of PCR product P5 by the use of the 5′-biotinylated primer LLP1, enabling the interaction between streptavidin and biotin usable for solid phase immunomagnetic separation [22,23] of the two DNA strands. The desired DNA strand, P7, which contains the cDNA of the target RNA, remained in the solution after immunomagnetic separation [**Fig. 2(b, c)**].

It is not possible to predict whether natural RNAs contain Nt.BstNBI and *Nla*III recognition sequences. If there is an Nt.BstNBI or *Nla*III recognition sequence inside a target RNA, the double-stranded DNA region covered cDNA of the target RNA can form an enzyme recognition sequence, therefore, cDNA of the target RNA may be digested by the restriction enzyme. To avoid cutting the cDNA of the target RNA, a stem-loop linker, SL2, was designed, which contained the recognition sequences of Nt.BstNBI and *Nla*III and the 5′-overhang of SL2 was based on SL1. After annealing SL2 with P7, SL2 restrictively paired with the complementary sequence of P7 (**Fig. 1**, **Table 1**). The stem-loop structure made precise pairing and guaranteed the formation of the sole restriction site on the cDNA strand for Nt.BstNBI and *Nla*III, which was just before the start of the 5′-terminus of the cDNA of target RNAs. This strategy not only prevents the interference from special RNAs that contain Nt.BstNBI and *Nla*III recognition sequences, but also insures unbiased amplification of RNA samples.

If it is necessary to ligate the nick at the junction between the two DNA fragments (P8) (**Fig. 1**), 5′-phosphorylated P7 is necessary. P7 could be 5′-phosphorylated by T4 polynucleotide kinase or using 5′-phosphorylated SLP1 primer for PCR.

Enzymatic digestion by *Nla*III and Nt.BstNBI

Restriction enzymatic cleavage of P8 by *Nla*III or Nt.BstNBI was assessed. Both ligated P8 [**Fig. 2(c)**] and nicked P8 [**Fig. 2(d)**] were compatible for enzymatic cleavage, however, these three restriction fragments [**Figs. 1** and **2(d)**, lane 3] produced from nicked P8 were more readily recognized and separated than the two enzymatic fragments produced from ligated P8. After digestion of

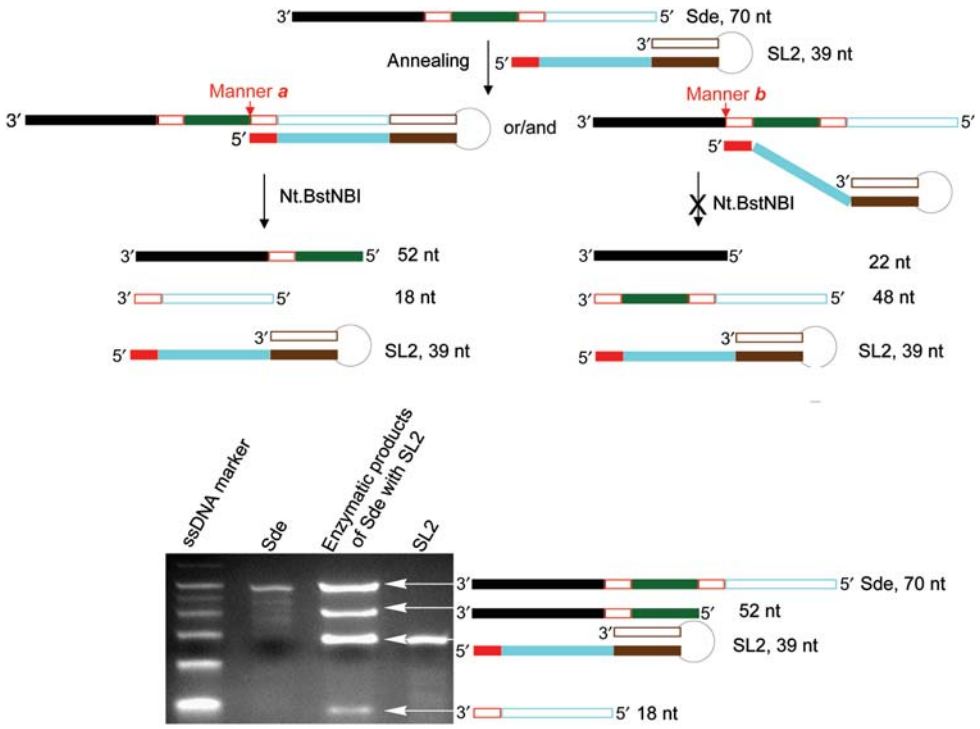

Figure 3 Schematic diagrams of possible enzymatic cleavage (upper) and gel electrophoretic analysis (lower) of annealing products of Sde and SL1 Red rods represent recognition sequences of Nt.BstNBI, sequences depicted by solid and hollow shapes are complementary and small arrowheads indicate cleavage sites; upper drawing depicts two possible enzymatic cutting modes of (a) and (b); lower diagram illustrates the gel electrophoretic results of enzymatic digestion analyzed by 15% 8 M urea denaturing PAGE. ssDNA marker: from the up 96, 70, 60, 50, 40, 30 and 20 nt, each band was indicated with a corresponding illustration.

nicked P8 by Nt.BstNBI, the desired product, P9 [second band from the top of lane 3 in **Fig. 2(d)**], was successfully produced. The sequence of P9 was determined (**Supplementary Fig. S1**) as predicted exactly. The site-specific restriction cutting by *Nla*III was less efficient compared with that of Nt.BstNBI, because the bases flanking the recognition sequence were necessary for *Nla*III cleavage (**Supplementary Fig. S2**).

Annealing of two recognition sequence DNA with stem-loop linker and enzymatic digestion by *Nla*III and Nt.BstNBI

After above experiments, we also inspected if the annealing and cutting were exclusive in the case of one or more recognition sequences inside an unknown RNA. Sde with two recognition sequences (red rods in **Fig. 3**, **Table 1**) for Nt.BstNBI and *Nla*III was designed and synthesized for the purpose. For the two recognition sequences in Sde, Sde possibly pairs with SL2 in two different manners (**Fig. 3**) to afford two possible enzymatic cleavage results. After annealing of Sde with SL2, Nt.BstNBI and *Nla*III were applied and enzymatic products revealed that the reaction by Nt.BstNBI proceeded efficiently in manner *a* not in manner *b* (**Fig. 3**). Thus, recognition sequence/s inside unknown RNAs did not interfere with pairing and enzymatic cutting of SL2 and cDNA.

Transcription of target RNA

Run-off transcription [24] by T7 RNA polymerase was performed using the T7P. To enhance the transcription efficiency and to discern target RNA from other fragments, the recessed end of the annealing product P10 was filled in using the Klenow fragment to form blunt-ended P11 [**Figs. 1** and **2(e)**]. Purified P11 was then transcribed to produce amplicons of target RNA [**Fig. 2(f, g)**]. Except for an extra G incorporated on the 5′-terminus and sometimes one additional C or A on the 3′-terminus [24,25], the amplified RNA is the same as the original natural target RNA.

Amplification *in vitro* of hsa_piR_021065

Recently, there are many reports investigating the biogenesis and the potentially diverse functions of piRNAs [5–9]. piRNAs are about 30 nt in length and play important roles in spermatogenesis by binding to mammalian Piwi proteins. In order to demonstrate that our RNA amplification method is suitable to amplify piRNA, a piRNA with 30 nt, hsa_piR_021065 was synthesized for our study [21]. The hsa_piR_021065 was replicated and amplified by RT-PCR after attachments of 5′ adapter and 3′ accepter, and then its cDNA strand was separated from PCR product. After exclusively pairing with SL2 and enzymatic cutting by Nt.BstNBI, at the end, run-off transcription

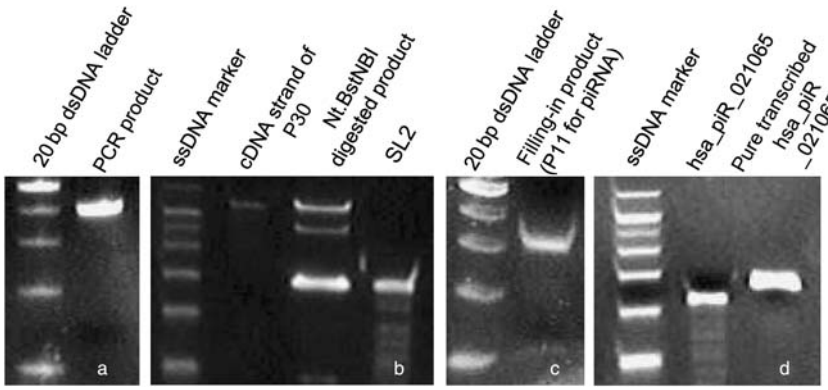

Figure 4 Gel electrophoretic analysis of amplification *in vitro* of hsa piR 021065 (30 nt) (a, c) 8%, native PAGE; (b, d) 15%, 8 M urea PAGE. ssDNA marker: from the up 96, 70, 60, 50, 40, 30 and 20 nt; 20 bp dsDNA ladder: from the up 100, 80, 60, 40 and 20 bp.

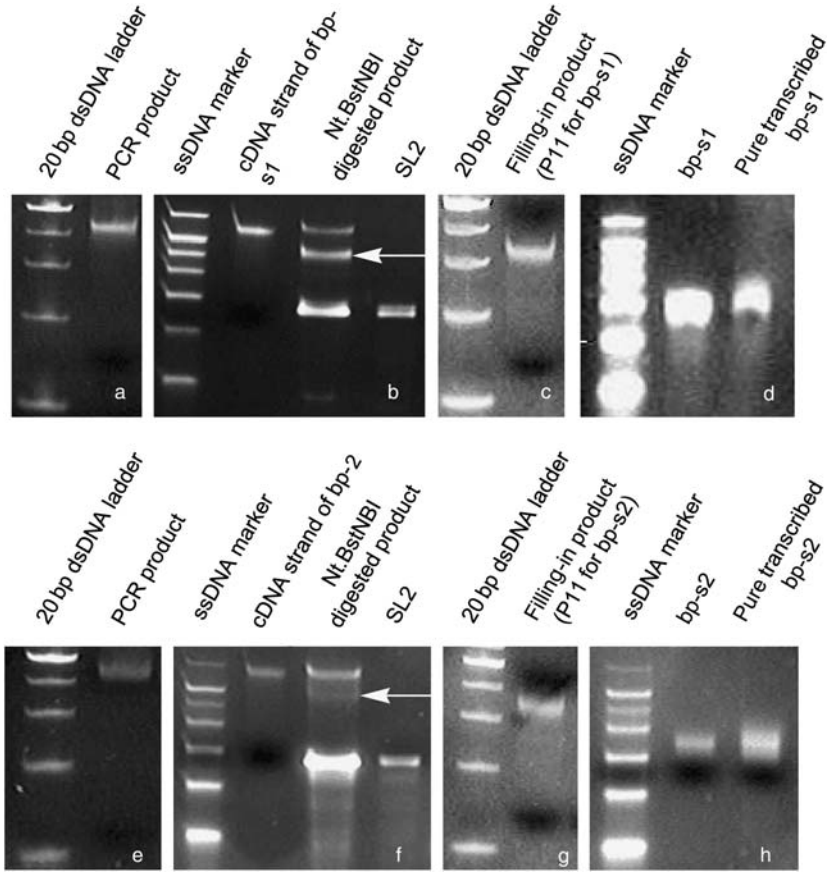

Figure 5 Gel electrophoretic illustrations for amplification *in vitro* of bp-s1 (a–d) and bp-s2 (e–h) (a, c, e, g) 8%, native PAGE; (b, d, f, h) 15%, 8 M urea PAGE. ssDNA marker: from the up 96, 70, 60, 50, 40, 30 and 20 nt; 20 bp dsDNA ladder: from the up 100, 80, 60, 40 and 20 bp.

in vitro of cDNA of hsa_piR_021065 generated the target RNA of hsa_piR_021065 (Fig. 4).

In vitro amplification of two small RNA mixtures from tumor-adjacent tissue of a liver cancer patient

We also amplified small RNA mixtures that were isolated from tumor-adjacent tissue of a liver cancer patient. Briefly, small RNAs (<200 nt in length) were extracted from tumor-adjacent tissue and then isolated small RNAs were analyzed on 15% denaturing PAGE. Two bands of bp-s1 (~33 nt) and bp-s2 (~40 nt) were cut out and extracted (**Supplementary Fig. S3**). To prevent self-circularization of bp-s1 and bp-s2, samples of bp-s1 and bp-s2 were pretreated with CIAP to remove the 5′-phosphate before its ligation with the 5′ adaptor. According to the same procedure as above, we successfully amplified bp-s1 and bp-s2 (Fig. 5).

Discussion

Most small RNAs that have been reported were cloned from different tissues and organisms using various cloning strategies [26–28], but the functions of most of them are unknown or unclear; many studies are focusing on individual small RNAs that are correlated with specific phenotypes during different developmental processes. Because it is not easy at the early research stage to identify functional small RNAs from the profile of an RNA sample, which has hundreds of different small RNAs, the generation of sufficient RNAs *in vitro* with the same fidelity as the original small RNA sample will greatly benefit downstream studies.

On the basis of the exclusive restriction enzyme site formed by a stem-loop linker, we have developed a global method to amplify target RNA(s). Introduction of a unique stem-loop linker into the cDNA strand is the key step to make a unique double-stranded enzymatic recognition sequence, which does not cover the cDNA sequence of the target RNA. The amplified RNA (except for an extra 5′ G and sometimes one additional 3′ A or C) is fully faithful to the original RNA sample in sequence quality. Because there is no bias in any protocol step, the RNA mixture can be precisely amplified with respect to sequence quality. The RNA sample to be amplified can be from various origins and can be a known or an unknown small RNA or a mixture of small RNAs. This amplification method is a useful and complementary technique in small RNA technology. With the technique, we are able to amplify our valuable and rare RNA samples for subsequent experimentation.

Supplementary data

Supplementary data is available at *ABBS* online.

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