

Capping-RACE: a simple, accurate, and sensitive 5' RACE method for use in prokaryotes

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ABSTRACT

Rapid amplification of cDNA ends (RACE) is a prevalent technique used to obtain the 5' ends of transcripts. Several different 5' RACE methods have been developed, and one particularly simple and efficient approach called CapFinder relies on the 5' cap-dependent template-switching that occurs in eukaryotes. However, most prokaryotic transcripts lack a 5' cap structure. Here, we report a procedure to capture primary transcripts based on capping the 5' triphosphorylated RNA in prokaryotes. Primary transcripts were first treated with vaccinia capping enzyme to add a 5' cap structure. First-strand cDNA was then synthesized using Moloney murine leukaemia virus reverse transcriptase. Finally, a template-switching oligonucleotide with a tail containing three ribonucleic acid guanines was hybridized to the cDNA 3' poly(C) and further used as template for reverse transcriptase. It is oligonucleotide sequence independent and is more sensitive compared to RLM-RACE. This approach specifically identified the transcription start sites of *ompA*, *sodB* and *shiA* in *Escherichia coli* and of *ompA*, *rne* and *rppH* in *Brucella melitensis*. Furthermore, we also successfully identified the transcription start sites of small noncoding genes *ryhB* and *micC* in *E. coli* and *bsnc135* and *bsnc149* in *B. melitensis*. Our findings suggest that Capping-RACE is a simple, accurate, and sensitive 5' RACE method for use in prokaryotes.

INTRODUCTION

Bacteria respond to a variety of environmental conditions by manipulating the expression of related genes, and the regulation of transcripts plays an important role in this process. The 5' untranslated region (5' UTR) extending from the transcription start site (TSS) to the start codon is crucial for mRNA stability and translational efficiency (1). There-

fore, the accurate identification of TSSs is indispensable for characterizing the structure and function of genes.

Rapid amplification of cDNA ends (RACE) is a novel technique used to determine the 5' end sequences of transcripts (2). In classic RACE, a homopolymer tail that acts as an anchor sequence can be added to the 3' end of cDNA by terminal deoxynucleotidyl transferase (TdT). The 5' terminal information is then obtained by PCR using a gene-specific primer (GSP) and an anchored primer complementary to the anchored sequence (2–4). Sequence- and ligation-independent cloning PCR (SLIC-PCR) and ligation-anchored PCR (LA-PCR) were subsequently developed to circumvent the nonspecific amplifications caused by using primers containing the anticomplementary sequence of the homopolymeric tail (5,6). These two methods are based on the ligation of a single-stranded modified oligonucleotide to the 3' end of first-strand cDNA, catalysed by T4 RNA ligase. However, the 5' ends determined with these methods might not be the actual transcription start sites because reverse transcriptases are subject to premature termination (7).

Another strategy for reducing nonspecific amplifications, known as RNA ligase-mediated amplification of cDNA ends (RLM-RACE), was also developed (7,8). In RLM-RACE, the full-length RNA strands are treated by tobacco acid pyrophosphatase (TAP) to remove the 5' cap structure and pyrophosphate, and then a RNA adaptor is directly ligated to the RNA 5' ends using T4 RNA ligase prior to reverse transcription. However, the RNA truncated at the 5' end can also be ligated to the RNA adaptor in this method. To address this limitation, an alternate method, oligo-capping, was developed. This approach works by removing the 5' pyrophosphate of non-capped RNAs to leave a hydroxyl before TAP treatment; thus, only the 5' ends of capped RNAs can be obtained using this method (9).

The methods described above all have multiple enzymatic steps, making them very time-consuming to perform, and also require quite large amounts of starting RNA. The CapFinder RACE method was designed to address these limitations (10,11). This procedure relies on the ability of the Moloney murine leukaemia virus (M-MLV) reverse transcriptase to add two to four cytosine residues to the 3'

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end of newly synthesized cDNA upon reaching the 5' cap structure of mRNAs. A template-switching oligonucleotide (TSO) containing a 3' poly(rG) tail is added to base pair with the extra cytosine residues, and then the TSO serves as a template for reverse transcription. As a result, a known adaptor is attached to the 3' terminus of the cDNA. To enhance the specificity and efficiency of this approach, various additional techniques, such as step-out PCR, inverse PCR and T-RACE, have been devised (12–14).

Notably, most of the methods mentioned above were developed for the mapping of eukaryotic transcripts. Prokaryotic RNA generally lack the 5' cap structure, which is essential for the application of CapFinder RACE (10,11). Furthermore, prokaryotic transcripts also lack a 3' poly(A) tail, which is used as a reverse transcription primer in eukaryotic RACE (15). Thus, the enrichment of primary transcripts in prokaryotes has been a great challenge. Recently, a novel approach called dRNA-seq was established (16,17). This protocol can enrich primary transcripts containing 5' triphosphates by utilizing terminator exonuclease (TEX), which specifically eliminates 5' monophosphorylated RNA. dRNA-seq has been widely applied to the genome-wide mapping of TSSs in different organisms and has even been applied to TSS mapping of the barley chloroplast (18–25). In addition, the Cappable-seq method has been reported as an alternative strategy to isolate primary transcripts in high-throughput sequencing (26,27). Unlike dRNA-seq, which removes unnecessary RNAs, in Cappable-seq, the 5' triphosphorylated RNAs are capped with biotinylated GTP using the vaccinia capping enzyme (VCE); the capped RNAs are then captured on streptavidin-coated magnetic beads and separated from other RNA.

Here, we describe a simplified 5' RACE method to precisely determine the TSSs of specific genes in prokaryotes that is based on the addition of a 7-methylguanylate cap structure to the 5' end of triphosphorylated RNA using VCE. In this approach, the 3' ends of cDNAs are obtained by template-switching as in CapFinder (10,11) and are then amplified by nest-PCR. Using this new method, we successfully identified the TSSs of protein-coding genes and non-coding genes in *Escherichia coli* and *Brucella melitensis*, suggesting that the 5' end sequences of other prokaryotic transcripts could be readily mapped by this Capping-RACE procedure.

MATERIALS AND METHODS

Primers

The complete list of primers used in this study is provided as Supplementary Material (Supplementary Table S1).

Bacterial strains and growth conditions

Escherichia coli strain MG1655 was cultured in Luria–Bertani (LB) medium. *Brucella melitensis* strain 16M was grown in tryptic soy broth (TSB) in a Biosafety Level 3 laboratory as previously described (28). Bacteria were grown at 37°C, 180 rpm to an OD₆₀₀ of 0.5–0.6. Growth of bacterial culture (5 ml per sample) was stopped by adding 0.625 ml of pre-chilled stop solution (5% phenol/95% ethanol).

Cells were harvested by centrifugation for 10 min at 6000 × g, and the resulting pellet was stored at –80°C.

RNA extraction and DNase I treatment

Frozen cell pellets were thawed on ice and resuspended in 100 µl per pellet of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH8.0). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA samples were then subjected to treatment with DNase I (TaKaRa, Dalian, China) to remove genomic DNA. The concentration and quality of each sample was evaluated using a Nano Drop (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis.

In vitro RNA transcription and purification

The template for the 111 nt *in vitro* transcription RNA was amplified from pwtCas9-bacteria obtained from Addgene (#44250) by forward primer 5'-CAGTAATACGACT CACTATTAGAAGCGACTCGTCTCAAACGG-3', which contains a T7 φ2.5 promoter, and reverse primer 5'-AGGATGACGTTTCATGCTTCTTGT-3'. The templates for A8_{AGAA}, G8_{GGAA}, G8_{GAAA}, G8_{GUAA}, G8_{GCAA}, G0, G1, G2, G3 and G4 were amplified by pairs of partially complementary oligodeoxynucleotides (Supplementary Table S1). The *in vitro* transcribed RNAs were generated with a T7 High Efficiency Transcription Kit (TransGen Biotech, Beijing, China). The 111 nt *in vitro* transcription RNA was purified using a Zymo Concentrator-5 kit (Zymo Research, Irvine, CA, USA), all other RNAs were purified by ethanol precipitation.

Different treatments of the 111 nt *in vitro* transcribed RNA

The 111 nt *in vitro* transcribed RNA was pre-treated to modify the 5' ends as follows: (i) 1 µg of RNA was incubated with 1 µl of RNA 5' pyrophosphohydrolase (RppH) (NEB, Ipswich, MA, USA) for 1 h at 37°C; (ii) 1 µg of RNA was incubated with 1 µl of VCE (NEB) for 30 min at 37°C; or (iii) 1 µg of RNA was treated with RppH for 1 h at 37°C and purified by ethanol precipitation, followed by incubation with VCE for 30 min at 37°C. All samples were purified by ethanol precipitation and evaluated using a Nano Drop (Thermo Fisher Scientific).

Capping of total bacterial RNA

A total of 1–10 µg of bacterial RNA was treated with 1 µl of VCE (NEB) as described above for 5' RACE, and the treated RNA was then purified by ethanol precipitation.

Capping-RACE cDNA synthesis

The cDNA synthesis was performed in two steps as previously described (29). A total of 0.5 µg of RNA and 2 pmol of gene-specific primer 1 (GSP1) were incubated at 65°C for 5 min and then quickly chilled on ice for 2 min before the addition of the following remaining components in a total volume of 10 µl: 1 µl of 10 mM dNTPs, 2 µl of 5 × buffer, 0.5 µl of DTT and 0.5 µl of 200 U SuperScript III Reverse

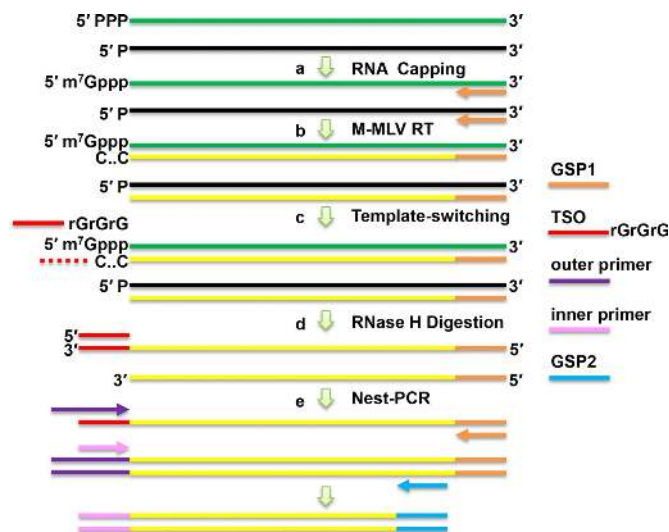


Figure 1. Schematic representation of the Capping-RACE procedure. (A) Total RNA is treated with vaccinia capping enzyme (VCE), which caps only the 5' triphosphorylated RNA. (B) First-strand cDNA synthesis is performed using M-MLV reverse transcriptase with a gene-specific primer (GSP1). (C) A template switching oligonucleotide (TSO) with a 3' end composed of three ribonucleic acid guanines hybridizes to the cDNA 3' poly(C) and is used as a template for reverse transcriptase. (D) Hybrid RNA is removed by RNase H. (E) Nest-PCR is performed using two sets of primers. The PCR products are cloned into pMD18-T vectors, which are sent out for commercial sequencing.

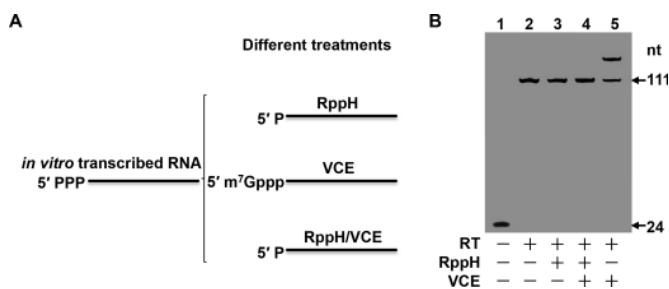


Figure 2. Experimental verification of Capping-RACE with *in vitro* transcribed RNA. (A) Different treatments of *in vitro* transcribed RNA. (B) The cDNA products from the differently treated RNA described in (A). Lane 1, the negative control, performed without adding reverse transcriptase (RT). Lane 2, *in vitro* transcribed RNA that was not subjected to any treatment. Lane 3, *in vitro* transcribed RNA subjected to RppH treatment. Lane 4, *in vitro* transcribed RNA subjected to dual treatment, i.e. RppH treatment prior to VCE treatment. Lane 5, *in vitro* transcribed RNA subjected to vaccinia capping enzyme (VCE) treatment. The reaction products were analysed on a 12% non-denaturing polyacrylamide gel and detected by a fluorescence image analyser (FUJIFILM, FLA-5100).

Transcriptase (Invitrogen). After incubation at 50°C for 60 min, 10 μ l of a mixture of 2 μ l of 0.1% bovine serum albumin (BSA), 0.8 μ l of 50 mM MnCl₂, 1 μ l of TSO, 2 μ l of 5 \times buffer, 0.5 μ l of DTT, 0.5 μ l of 200 U SuperScript III Reverse Transcriptase, and 3.2 μ l DEPC-treated water was added to each sample. The combined reaction was then incubated at 42°C for an additional 90 min. Finally, the reverse transcriptase was inactivated by heating the samples at 70°C for 10 min.

RLM-RACE

RLM-RACE was performed as described previously with modification (7–9). Briefly, bacterial RNA was treated with 1 μ l of RNA 5' pyrophosphohydrolase (RppH) (NEB, Ipswich, MA, USA) for 1 h at 37°C, and purified by ethanol precipitation. The treated RNA was ligated to a synthetic RNA adaptor with T4 RNA ligase 1 (Thermo Fisher Scientific) for 30 min at 37°C. The ligation product was reverse transcribed with 2 pmol of gene-specific primer 1 (GSP1) by SuperScript III Reverse Transcriptase (Invitrogen) for 1 h at 55°C and 10 min at 70°C. The cDNA was amplified by nest-PCR. Finally, the PCR products were detected by agarose gel electrophoresis.

Nest-PCR and sequencing

For amplification of the 5' end sequences, nest-PCR was performed using multiple sets of primers. Briefly, the first-round PCR was carried out using the outer primer and GSP1. The outer primer contains a part of the TSO. The resulting product was then diluted 100-fold before being used as the template for the second-round PCR, which was performed using the inner primer and GSP2. The inner primer is identical to the 3' end of the outer primer. The final PCR products were separated by agarose gel electrophoresis and purified from the gel using a Universal DNA Purification Kit (Tiagen, Beijing, China). Finally, the purified product was cloned into pMD18-T vector (Takara), and the resulting clones were sequenced.

RESULTS

General strategy for Capping-RACE

The overall strategy for the Capping-RACE approach is illustrated in Figure 1. The basic principle of this method is the treatment of total RNA with VCE to add a cap structure to the end of 5' triphosphorylated RNA (Figure 1A). The cDNA synthesis by M-MLV reverse transcriptase begins with a gene-specific primer (GSP1) (Figure 1B). In the presence of MnCl₂ and BSA, the reverse transcriptase adds a few cytosines to the 3' end of the cDNA when reaching the cap structure; this allows cDNA generated by the capped RNAs to be hybridized to a TSO adaptor containing a 3' poly(rG) tail (Figure 1C). The cDNA polycytosine end synthesized by RT continues to extend in response to the TSO. RNase H is then used for the removal of complementary RNA to enhance the PCR efficiency (Figure 1D). Next, nest-PCR is performed using two sets of primers (Figure 1E). Finally, the PCR products are cloned into a pMD18-T vector, and the products are sent away for sequencing.

Verification of the feasibility of Capping-RACE

To determine whether the Capping-RACE method can be used to map primary transcripts, we conducted experiments on a 111 nt *in vitro* transcribed RNA that has a 5' triphosphorylated end. The *in vitro* transcribed RNA was pre-treated to modify the 5' end in one of three ways (Figure 2A). In pre-treatment 1, the RppH treatment removes pyrophosphate from the RNA to leave a 5' monophosphate

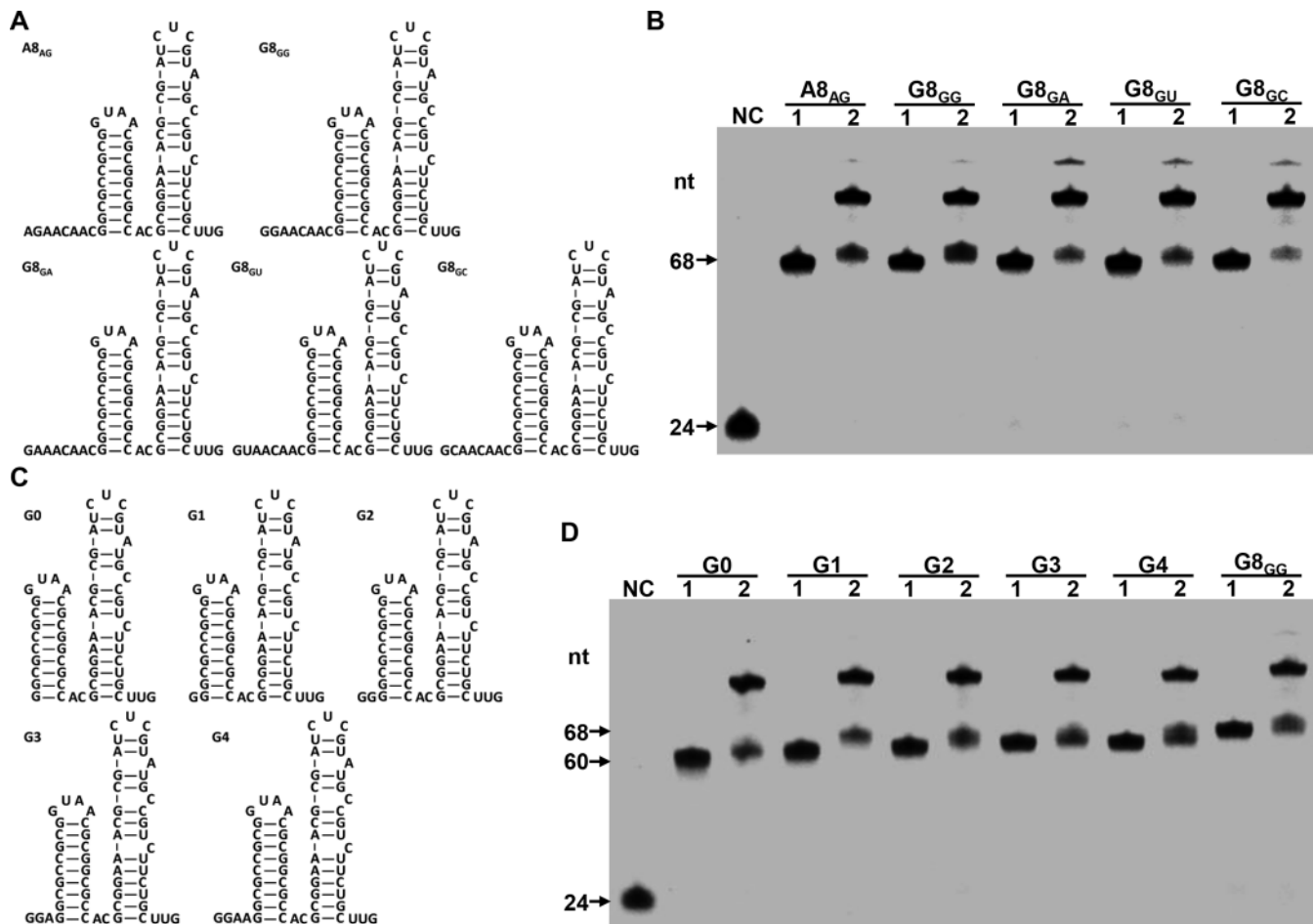


Figure 3. Determination of the Capping-RACE efficiency for transcripts with different 5' terminal sequence. (A and C) The sequence and secondary structure of *in vitro* transcribed RNAs are shown. The letter indicates the 5' terminal nucleotide, and the numeral indicates the number of unpaired nucleotides at the 5' end. (B and D) The cDNA products from *in vitro* transcribed RNAs. Lane NC, the negative control, FAM-RT primer 2 on the 5' end using 6-carboxy fluorescein phosphoramidite. Lane 1 and lane 2, Capping-RACE for the *in vitro* transcribed RNAs without or with TSO.

RNA. In pre-treatment 2, the VCE treatment adds a cap structure to the 5' end of the RNA. Pre-treatment 3 was a dual-treatment in which RppH treatment was performed prior to VCE treatment; this served as a control. The first-strand cDNA synthesis was then initiated with the FAM-RT primer 1 modified on the 5' end using 6-carboxy fluorescein phosphoramidite. As expected, template-switching did not occur for the 5' triphosphorylated and 5' monophosphorylated RNAs that lacked cap structures (Figure 2B, lanes 2 and 3). Since RppH-treated RNA cannot be capped, the dual-treated RNA was also not attached to a TSO adaptor (Figure 2B, lane 4). About 60% of the capped RNA was attached by the TSO adaptor (Figure 2B, lane 5). These results suggest that Capping-RACE is feasible for mapping the 5' end of transcribed RNAs.

Capping-RACE is sequence independent

The data described above (Figure 2) indicated that Capping-RACE could be a simplified 5' RACE method. To investigate whether Capping-RACE has a bias for 5' terminal sequence of transcripts, we designed several *in vitro* transcribed RNAs which had known nucleic acid sequence

and secondary structure, referring to reported oligonucleotides with modifications (30). All the RNAs comprised an 8-nucleotide single-stranded segment and two stem-loop structures (Figure 3A). We tested the efficiency of Capping-RACE for these RNAs. The first-strand cDNA synthesis was initiated with the FAM-RT primer 2 modified on the 5' end using 6-carboxy fluorescein phosphoramidite. As shown in Figure 3B, all the RNAs were capped and subsequently ligated to the TSO with a high yield (>50%).

Next, to determine the effect of complex structures on Capping-RACE, we synthesized a set of G8_{GG} variants by truncating the length of single-stranded segment (G0, G1, G2, G3 and G4; Figure 3C). These variants have only 0 to 4 unpaired nucleotides at the 5' end. Then we applied Capping-RACE to these variants. As anticipated, the effectiveness of Capping-RACE still exceeded 50% for each RNA (Figure 3D). These results gave further support to the utility of Capping-RACE for prokaryotic transcripts.

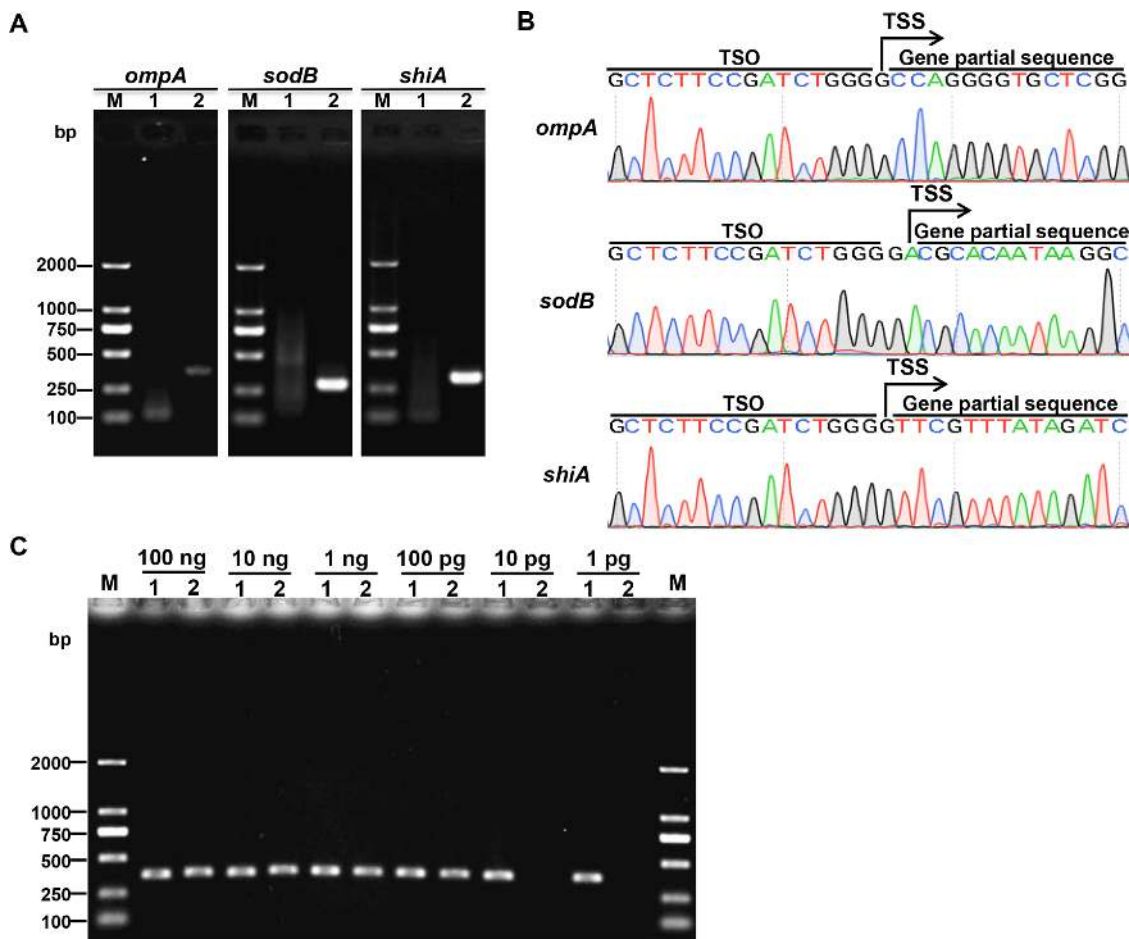


Figure 4. 5' RACE mapping of *E. coli* MG1655 protein-coding genes. (A) Nest-PCR products of 5' RACE starting from *E. coli* MG1655 total RNA. *ompA*, outer membrane protein A (371 bp); *sodB*, superoxide dismutase (294 bp); *shiA*, shikimate transporter (320 bp). Lane M, DL2000 DNA Marker (Takara); lane 1, first-round PCR products; lane 2, second-round PCR products. (B) The sequencing result of the second-round PCR products. (C) The second-round PCR products of Capping-RACE and RLM-RACE for *ompA*, starting from different input of total RNA. Lane M, DL2000 DNA Marker (Takara); lane1, Capping-RACE; lane2, RLM-RACE.

The application and reproducibility of Capping-RACE for selected examples

To test Capping-RACE in prokaryotes, we selected three protein-coding genes, *ompA*, *sodB* and *shiA*, of the model organism *E. coli* MG1655 as targets. The resulting PCR products are shown in Figure 4A. The specific second-round PCR products were purified from the gel and sequenced (Figure 4B). The TSS information acquired by Capping-RACE is in accordance with the experimental data published previously (31). To ascertain the sensitivity of Capping-RACE, we used a variety of starting input of total RNA to identify the TSS of *ompA*. Simultaneously, RLM-RACE was carried out for comparison. The result showed that only 1 pg of total RNA was required for Capping-RACE, however, RLM-RACE need at least 100 pg of total RNA (Figure 4C). The data suggest that Capping-RACE is more sensitive than RLM-RACE.

For further validation of this method in another prokaryotic species, we applied Capping-RACE to identifying the TSSs of protein-coding genes of *B. melitensis* 16M, which is an important facultative intracellular pathogen. As shown

in Figure 5, accurate TSSs were identified by the Capping-RACE data. These results confirm that Capping-RACE could be used for TSS identification of prokaryotic protein-coding genes.

Capping-RACE of prokaryotic non-coding genes

We also selected two sRNAs from each of the above two bacterial species. *RyhB* and *micC* are two of the most important sRNAs of *E. coli*, and *bsnc135* and *bsnc149* are two sRNAs of *B. melitensis* 16M that were identified previously (32). As expected, the 5' ends were detected successfully (Figures 6 and 7), and all the TSS information obtained was in accordance with published data (30,32). The results reveal that Capping-RACE can be applied not only to protein-coding genes but also to non-coding genes.

DISCUSSION

RNA-seq has rapidly become a powerful tool for understanding gene sequence and structure as well as RNA-based regulation in diverse organisms. However, RNA-seq is an

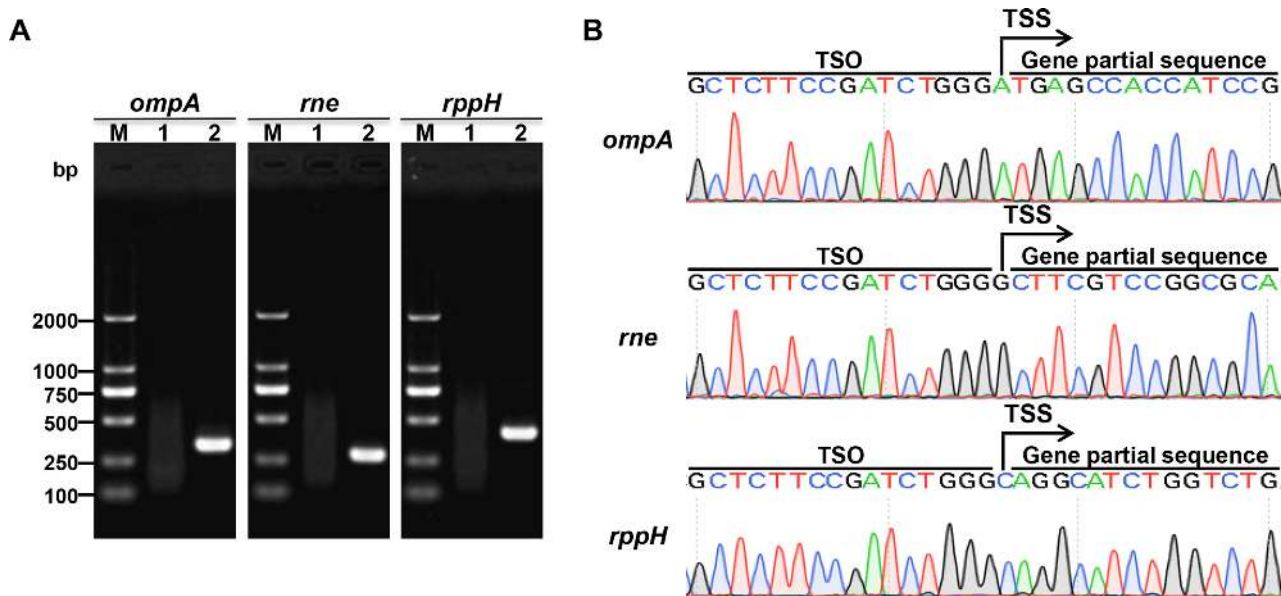


Figure 5. 5' RACE mapping of *B. melitensis* 16M protein-coding genes. (A) Nest-PCR products of 5' RACE starting from *B. melitensis* 16M total RNA. *ompA*, outer membrane protein A (337 bp); *rne*, an endoribonuclease (286 bp); *rppH*, RNA pyrophosphohydrolase (400 bp). Lane M, DL2000 DNA Marker (Takara); lane 1, first-round PCR products; lane 2, second-round PCR products. (B) The sequencing result of the second-round PCR products.

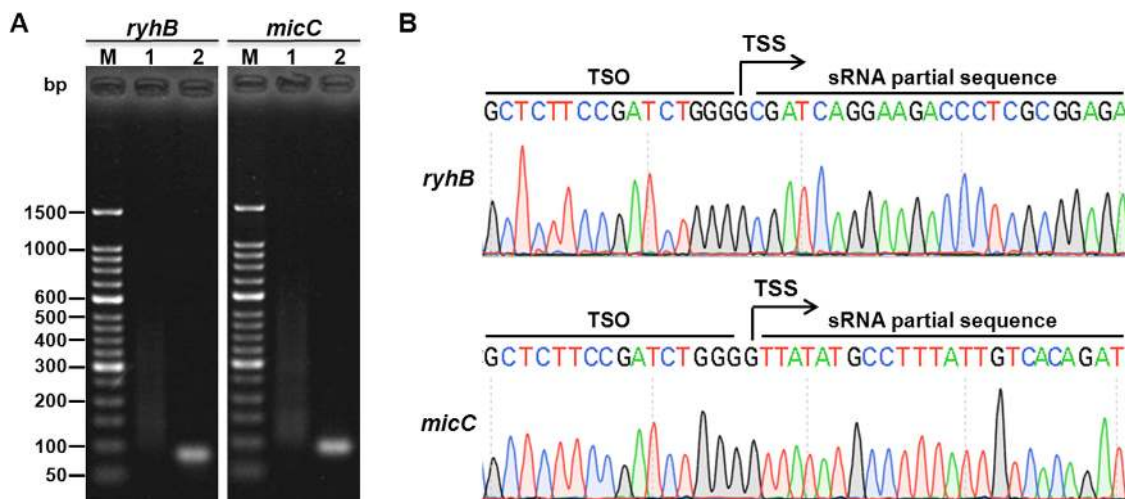


Figure 6. 5' RACE mapping of *E. coli* MG1655 non-coding genes. (A) Nest-PCR products of 5' RACE of two *E. coli* MG1655 sRNAs, *ryhB* (85 bp) and *micC* (93 bp). Lane M, 50 bp DNA Ladder (Takara); lane 1, first-round PCR products; lane 2, second-round PCR products. (B) The sequencing result of the second-round PCR products. The positions of the TSSs are indicated by black arrows.

extremely complex and time-consuming method because it requires the construction of a cDNA library followed by sequencing and data analysis. This technique is mainly suitable for comprehensive transcriptome analyses. Currently, dRNA-seq and Cappable-seq provide approaches for genome-wide mapping of TSSs (16,17,26,27). However, RACE is still an efficient technique for precisely obtaining the 5' end sequence of specific transcripts. Since most prokaryotic transcripts lack a 5' cap structure and a 3' poly(A) tail, the previous RACE methods that were developed for use with eukaryotic transcripts cannot be used effectively for prokaryotes.

In this study, we present a simplified 5' RACE strategy, i.e. Capping-RACE, for targeting genes in prokaryotes.

This approach adds a cap structure to any transcripts containing a 5' terminal triphosphate. The 5' ends of cDNAs are then obtained by template-switching in the same manner as in CapFinder (10,11). Our data show that this new method can be successfully applied to determining the 5' ends of both protein-coding and non-coding genes (Figures 4–7). Since this approach cannot add cap structure to transcripts terminated with 5' monophosphate, 5' hydroxyl or 5' NAD cap, the method is not suitable for these transcripts. Compared with previous procedures, our method has several advantages. It is more accurate and sensitive because it avoids many non-specific effects by capping the 5' triphosphorylated RNA. This method has been confirmed to be sequence independent (Figure 3). Furthermore, only

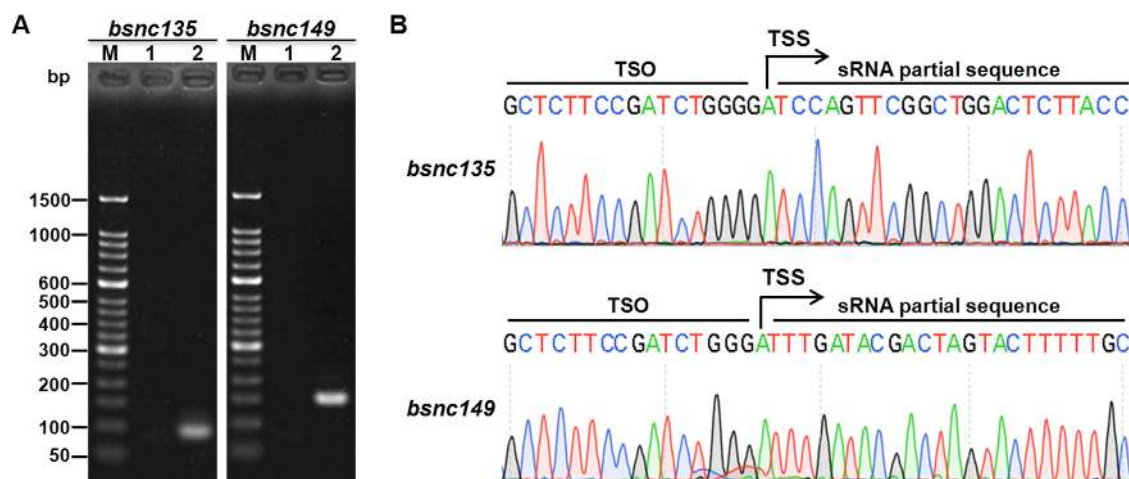


Figure 7. 5' RACE mapping of *B. melitensis* 16M non-coding genes. (A) Nest-PCR products of 5' RACE of two *B. melitensis* 16M sRNAs, *bsnc135* (92 bp) and *bsnc149* (155 bp). Lane M, 50 bp DNA Ladder (Takara); lane 1, first-round PCR products; lane 2, second-round PCR products. (B) The sequencing result of the second-round PCR products. The positions of the TSSs are indicated by black arrows.

a minimal amount of total RNA is required for template-switching, so this method requires less starting material than alternative techniques (Figure 4C). Our results suggest that Capping-RACE has significant application potential for the identification of TSSs of prokaryotic transcripts.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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