

## Rolling circle amplification-RACE: a method for simultaneous isolation of 5' and 3' cDNA ends from amplified cDNA templates

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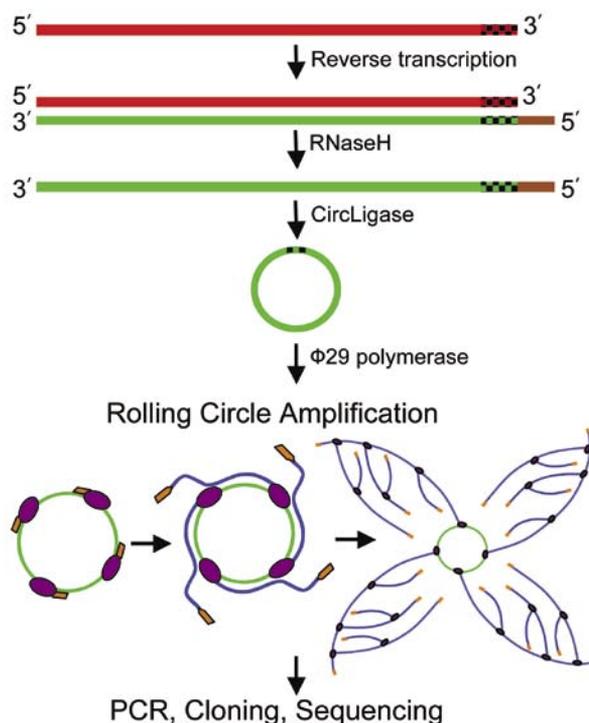
Isolation of full-length gene transcripts is important to determine the protein coding region and study gene structure. However, isolation of novel gene sequences is often limited to expressed sequence tags (ESTs) (i.e., short cDNA fragments that predominantly represent the 3' end of the transcript). Rapid amplification of cDNA ends (RACE) is today by far the most popular approach for obtaining full-length cDNAs when only part of the transcript's sequence is known.

Since its original description (1,2) numerous modifications and improvements of the method have been developed and consist of a collection of PCR-based cloning procedures that extend a known cDNA fragment toward the 3' (3' RACE) or the 5' (5' RACE) cDNA end. The original method is based on attachment of an anchor sequence to one end of the cDNA that can be used as a primer binding template in PCR with a second gene-specific primer from the known part of the gene. Although this procedure seems in theory fast and simple, it is technically difficult and usually requires substantial optimization and several repetitions before satisfactory results can be obtained (3). This is particularly due to the use of a universal primer corresponding to the anchor sequence present in all cDNAs, which may result in a high background of nonspecific products even after a nested PCR with a gene-specific primer internal to the first gene-specific primer is performed. Another drawback of the method is the difficulty of obtaining the full-length 5' end of the transcript due to the presence of many truncated transcripts in the messenger RNA

(mRNA) pool. Several strategies aimed at eliminating these problems have been developed (4–9) and have proven to be very useful in certain applications. One improvement is based on the utilization of a pair of gene-specific primers in inverse PCR on circularized cDNA templates, which would avoid the use of a universal primer and the problems it may generate (4,6,8–10). This strategy also allows the simultaneous isolation of both cDNA ends in a single reaction (9,10). Some of these procedures require the generation of double-stranded cDNA, including the use of template-switching reverse transcription (9) or a post-reverse

transcription adaptor ligation step (10). Methods that are performed directly on first-strand cDNA are complicated by the low efficiency of RNA ligase for the circularization reaction (6) or the need for bridging oligonucleotides for this step (8). Furthermore, existing inverse-RACE methods typically require nested PCR to amplify the transcript of interest, and only a limited number of transcripts can be isolated from a single reverse transcription reaction, making it difficult to analyze rare transcripts from scarce tissue.

We describe here an improved inverse-RACE method, which uses CircLigase™ (Epicentre Biotechnologies, Madison, WI, USA) for cDNA circularization, followed by rolling circle amplification (RCA) of the circular cDNA with  $\phi$ 29 DNA polymerase (New England Biolabs, Ipswich, MA, USA). In this way, a large amount of the PCR template is produced, allowing the simultaneous isolation of the 3' and 5' unknown ends of a virtually unlimited number of transcripts after a single reverse transcription reaction. Figure 1 illustrates this method, named RCA-RACE. The process takes advantage of the properties of CircLigase to circularize



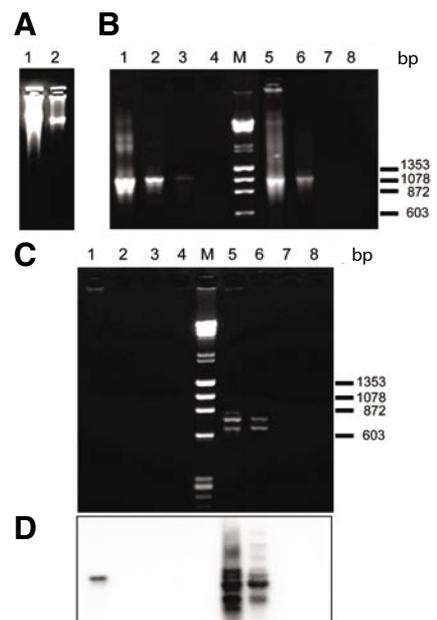
**Figure 1. The rolling circle amplification rapid amplification of cDNA ends (RCA-RACE) method.** Messenger RNA (mRNA; red) with a poly(A) tail (red checkerboard pattern) is reverse transcribed into cDNA (bright green) using an oligo(dT) (bright green checkerboard pattern) primer harboring a 5' phosphorylated adaptor (brown). After RNaseH treatment, the resulting cDNA is circularized using CircLigase. The circular cDNA is then amplified by RCA using  $\phi$ 29 DNA polymerase (violet oval) and random hexamer primers (orange) to multicopy concatemers (blue). For each transcript of interest, an aliquot of the RCA reaction serves as a template in an inverse PCR to obtain simultaneously the transcript's 5' and 3' ends.

single-stranded cDNA molecules via an intramolecular link. This ATP-dependent ligase can circularize single-stranded DNA (ssDNA) templates that have a 5'-phosphate and a 3'-hydroxyl group and are longer than 30 nucleotides. According to the manufacturer, under standard reaction conditions, the enzyme makes essentially no linear or circular concatemers, since it catalyzes only intramolecular ligations. In addition, although CircLigase is influenced by the ssDNA sequence, high concentrations of the enzyme can effectively circularize difficult templates (www.epibio.com/pdftechlit/222pl085.pdf). The circularized cDNA is then amplified in a RCA reaction using the  $\phi$ 29 DNA polymerase and random primers (11,12). This would allow the generation of enough template for the cloning of rare transcripts, as well as high-throughput cloning of cDNA ends for large numbers of genes from scarce tissue, which cannot be effectively performed with standard RACE methodologies.

To prove the concept of the proposed methodology, we used the procedure to isolate two previously characterized transcripts: (i) the H<sub>2</sub>O<sub>2</sub>-induced maize (*Zea mays*) *Aox1a* (GenBank® accession no. AY059647), which represents a relatively abundant transcript (13), and (ii) the crocus (*Crocus sativus*) AP1/FUL-like MADS-box gene *CsAPIa* (GenBank accession no. AY337928), which represents a rare transcript (14). Total RNA was extracted as described previously (13,14). First-strand cDNA was synthesized using 3  $\mu$ g RNA in a reaction containing 0.5  $\mu$ g oligo(dT)-adaptor primer [5'-GGC CACGCGTCTCGACTAGTAC(T)<sub>18</sub>-3'] phosphorylated at the 5' end, 0.5 mM dNTPs, 10 mM dithiothreitol (DTT), 1 $\times$  first strand buffer (Invitrogen, Paisley, UK), and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). The reaction was incubated at 37°C for 1 h, followed by heat inactivation of MMLV reverse transcriptase at 70°C for 15 min. After the addition of 2 U RNaseH (Invitrogen), the reaction was incubated at 37°C for 20 min and purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany).

Half of the purified cDNA (15  $\mu$ L) was circularized using 100 U CircLigase, 1 $\times$  reaction buffer (Epicentre Biotechnologies), and 50  $\mu$ M ATP at 60°C for 1 h, followed by inactivation of the enzyme at 80°C for 10 min and purification using the QIAquick PCR purification kit. The possibility of directly using the circular cDNA as an inverse PCR template for isolation of *Aox1a* and *CsAPIa* was examined, and the results indicated that additional steps were required for successful isolation of these transcripts (see the supplementary material available online at www.BioTechniques.com). For direct cloning of an unknown transcript, optimization of the PCR conditions might improve performance, and nested PCR with internal primers could be the most appropriate solution to avoid high background problems (Supplementary Figure S1). However, our results suggested that if a gene-specific probe is available, then selection of the longest hybridizing band for cloning could lead to isolation of the full-length transcript of the gene (see the supplementary material).

We amplified the circular cDNA pool in order to provide enough PCR template to enable high-throughput isolation of rare transcripts from a limited amount of tissue. We used an RCA method with the  $\phi$ 29 DNA polymerase to amplify the circular single-stranded cDNA pool.  $\phi$ 29 is a polymerase able to incorporate >70,000 nucleotides without dissociating from the template, has excellent strand displacement activity with linear reaction kinetics at 30°C for over 12 h, and is very stable, allowing high amplification rates (11,12). RCA reactions were performed in 50  $\mu$ L volume containing a 10- $\mu$ L aliquot of the circularized crocus or maize cDNA, 1 mM dNTPs, 200  $\mu$ g/mL bovine serum albumin (BSA; New England Biolabs), 1 $\times$   $\phi$ 29 DNA polymerase reaction buffer (New England Biolabs), 10 U  $\phi$ 29 DNA polymerase, and either 10  $\mu$ M random hexamers or 1  $\mu$ M InVUP primer (5'-GTACTAGTCTCGACGCTGGCC-3') both modified by the addition of two phosphothioate linkages on the 3' end (synthesized by VBC-Genomics, Vienna, Austria) to make the primers



**Figure 2. Rolling circle amplification (RCA) and inverse PCR on amplified templates.** (A) Electrophoresis of RCA reactions performed with the  $\phi$ 29 DNA polymerase and random hexamers (lane 1) or the InVUP primer (lane 2) on circularized single-stranded cDNA from maize. (B) Amplification products of *Aox1a* gene using the AoxF/InvR primers on serial dilutions of RCA reactions performed with the InVUP primer (lanes 1–4) or random hexamers (lanes 5–8). Lanes 1 and 5, undiluted template; lanes 2 and 6, 10<sup>-2</sup> template dilution; lanes 3 and 7, 10<sup>-4</sup> template dilution; lanes 4 and 8, 10<sup>-6</sup> template dilution; M,  $\lambda$ HindIII/ $\phi$ X174HaeIII molecular weight marker (Finnzymes). Relevant sizes in base pairs are shown at right, and the expected size of a product is approximately 1100 bp. (C) Amplification products of *CsAPIa* transcript using CsAPI-F/Inv1 primers on serial dilutions of RCA reactions performed with the InVUP primer (lanes 1–4) or random hexamers (lanes 5–8). Lanes 1 and 5, 10<sup>-1</sup> template dilution; lanes 2 and 6, 10<sup>-2</sup> template dilution. In this experiment, control PCRs were performed with template produced in RCA in which  $\phi$ 29 was omitted from the reaction to test if circularized, but not amplified, single-stranded cDNA could provide a suitable template for PCR. Lanes 3 and 4 represent the controls for lanes 1 and 2, respectively; similarly, lanes 7 and 8 are the controls for lanes 5 and 6, respectively. M indicates the  $\lambda$ HindIII/ $\phi$ X174HaeIII molecular weight marker, and relevant sizes in base pairs are shown at right. (D) Hybridization of the PCR products after transfer to a membrane with the *CsAPIa* probe. The visible gel bands (lanes 5 and 6), as well as a single band of the expected size (approximately 850 bp) not visible on the gel (lane 1), gave hybridization signals.

resistant to the  $\phi 29$  exonuclease activity. Control reactions containing all reagents except the  $\phi 29$  DNA polymerase were also performed. The reactions were incubated at 30°C for 21 h followed by heat inactivation at 60°C for 10 min, and RCA products were verified by electrophoresis. RCA was performed using either random hexamer primers or a cDNA-specific InvVUP primer complementary to part of the oligo(dT)-adaptor used for priming cDNA synthesis (the reactions are abbreviated as RCA-hexamer and RCA-InvVUP, respectively). Similar results from the RCA were obtained using the circularized cDNA pools generated from maize or crocus; representative results obtained from maize are shown in Figure 2A. The RCA-hexamer reaction yielded a smear of high molecular weight DNA, in accordance to previously described results (12). The RCA-InvVUP reaction yielded a distinct band of high molecular weight. This difference may reflect a fundamental distinction in the two approaches: the amplified molecules with random hexamers are a mixture of double-stranded DNA of different molecular weights, while the molecules that resulted from the InvVUP primer are uniform (dependent on the polymerase efficiency) high molecular weight ssDNA.

Templates from both the RCA-hexamer and RCA-InvVUP reactions were used in PCR to amplify the maize *Aox1a* and crocus *CsAPIa* transcripts. Inverse-PCR for obtaining the maize *Aox1a* sequence was performed using 0.5  $\mu$ L neat or serially diluted ( $10^{-2}$ ,  $10^{-4}$ , and  $10^{-6}$ ) RCA reaction as template, along with the AoxF (5'-GCTGCGGTGCCGGAATGGT-3') and InvR (5'-TGGACATAAGCCGTAGCATG-3') primers and DyNAzyme™ II DNA polymerase (Finnzymes, Espoo, Finland). The cycling parameters were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 56°C for 45 s, 72°C for 1.5 min, and a final extension step of 72°C for 10 min. A single clearly distinct band was observed in PCRs performed using either the hexamer- or InvVUP-primed templates; the size of this band was that expected from a full-length transcript (Figure 2B). For the RCA-hexamer template, the product band could be

observed in lanes corresponding to the neat template and the  $10^{-2}$  dilution (Figure 2B, lanes 5 and 6). For the RCA-InvVUP template, bands were detectable even for the  $10^{-4}$  template dilution (Figure 2B, lane 3). The bands of the  $10^{-2}$  dilution from both reactions were excised from the gel, cloned, and sequenced. Both contained the same approximately 1100-bp full-length sequence expected for the maize *Aox1a* transcript.

For obtaining the crocus *CsAPIa* sequence, two serial dilutions,  $10^{-1}$  and  $10^{-2}$ , of the RCA reaction were used as template in PCR performed with the Inv1 (5'-GCGCTTCGAGAAGGTGACCTG-3') and CsAPI-F (5'-AATGGCACAGCAGGGACACTG-3') primers. The cycling conditions were as described above with the difference that the annealing step was performed at 52°C, and the initial denaturation step was for 5 min. Results showed that 2–3 visible bands were present in the lanes corresponding to both dilutions of the RCA-hexamer template, and no band could be detected from the RCA-InvVUP template (Figure 2C, lanes 5–6 and 1–2, respectively). Control PCRs using templates produced in RCA reactions from which  $\phi 29$  had been omitted were included to test if circularized, but not amplified, single-stranded cDNA could provide suitable template for amplification. Results of these controls indicated that no visible products could be obtained (Figure 2C, lanes 3–4 and 7–8). Hybridization of the PCR products with the *CsAPIa* probe revealed that the visible bands were recognized by the probe (Figure 2D). In addition, a single band of the expected size (approximately 850 bp) was detected in the lane corresponding to PCR performed with the  $10^{-1}$  dilution of the RCA-InvVUP template (Figure 2D, lane 1). Comparing lanes 5 and 6 in Figure 2D, it is also evident that higher template concentrations increased PCR byproduct formation. The three bands from the RCA-hexamer template at the  $10^{-2}$  dilution (Figure 2C, lane 6) were excised from the gel, cloned, and sequenced. The approximately 850-bp band represented the expected sequence from a full-length crocus *CsAPIa* transcript. The two shorter bands

represented truncated products lacking part of the 5' end of the transcript. The RCA-RACE approach has also been successfully applied in our laboratory for isolation of novel transcripts from crocus (Kalivas and Tsaftaris, unpublished), peach (Tani, Polidoros, and Tsaftaris, unpublished), and cotton (Michailidis and Tsaftaris, unpublished), providing additional supportive evidence for the validity of the method.

Although a cDNA circularization/RCA procedure has been previously proposed (U.S. patent application no. 20050069939), that report focused on circularization by use of a bridging oligonucleotide and lacked a demonstration of the strategy's application to efficient isolation of cDNA ends. The RCA-RACE method we describe here is simple and requires no additional steps for second strand cDNA synthesis or adaptor ligation for RACE, and it uses a simple and robust circularization protocol. It is very efficient in isolating full-length transcripts, since it was demonstrated that only a single specific full-length PCR product or a few products, the longest of which is full-length, are produced. It is highly flexible since amplification of the original cDNA pool with RCA can be performed with random hexamers (leading to more efficient amplification of rare transcripts) or a cDNA-specific adaptor primer (resulting in increased specificity and low complexity of the amplified cDNA pool). RCA-RACE can be advantageous over standard RACE methods for high-throughput isolation of transcripts, for isolation of rare transcripts, simultaneous isolation of both ends of a transcript, and for isolation of genes whose available sequence is limited.

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### COMPETING INTERESTS STATEMENT

*The authors declare no competing interests.*

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