Chapter 8

Validation of Circular RNAs by PCR

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Abstract

High-throughput RNA-sequencing (RNA-seq) technologies combined with novel bioinformatic algorithms discovered a large class of covalently closed single-stranded RNA molecules called circular RNAs (circRNAs). Although RNA-seq has identified more than a million circRNAs, only a handful of them is validated with other techniques, including northern blotting, gel-trap electrophoresis, exonuclease treatment assays, and polymerase chain reaction (PCR). Reverse transcription (RT) of total RNA followed by PCR amplification is the most widely used technique for validating circRNAs identified in RNA-seq. RT-PCR is a highly reproducible, sensitive, and quantitative method for the detection and quantitation of circRNAs. This chapter details the basic guidelines for designing suitable primers for PCR amplification and validation of circRNAs.

Key words Divergent primer, Full-length primer, Rolling circle amplification, PCR, Sanger sequencing

1 Introduction

The advent of RNA-sequencing (RNA-seq) technologies and the novel computational pipelines discovered tens of thousands of circular RNAs (circRNAs) in various organisms, including humans [1–4]. CircRNAs are a large family of covalently closed single-stranded RNA molecules found to be ubiquitously expressed and conserved during evolution. Their size ranges from less than 100 nt to several thousand bases [3, 4]. CircRNAs are generated from the pre-mRNAs by a head-to-tail splicing mechanism known as back-splicing [5, 6]. CircRNAs are resistant to exonuclease and are very stable compared with the linear RNAs due to the lack of 5′ and 3′ ends [7–9]. CircRNAs have been shown to be involved in disease development by regulating various critical events in the cells. Recent evidence suggested their role in gene regulation by acting as a sponge for RNA-binding proteins and microRNAs [10]. The unique nonlinear backsplice junction sequence serves as the key for detecting circRNAs in the RNA-seq data [11, 12]. However, the backsplice junctions identified in the RNA-seq may come from

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reverse transcriptase template switching, trans-splicing, and backsplicing. Validation of true circRNA and their actual spliced sequence is an essential step for their functional characterization. Further validation of circRNAs can be achieved by various techniques, including exonuclease assay [13], in situ hybridization [14], northern blotting [15], and RT-PCR [16].

Since RT-PCR is a highly sensitive and versatile method, it has been widely adopted as the second most used technique for identifying and quantifying circRNAs after RNA-seq. However, detection and quantification of circRNAs require an accurate protocol for designing special primers and performing semiquantitative/quantitative (q)PCR. Unlike linear RNAs, circRNA detection requires outward-facing divergent primers that allow specific amplification of the backsplice junction sequence of target circRNA [16]. Here, we provide a detailed protocol for divergent primer design and the basic PCR technique followed by Sanger sequencing to validate the backsplice junction sequence obtained from RNA-seq data. Additionally, we describe the detailed method for RNase R treatment and RT-qPCR using divergent primers for validating the circular nature of the RNA molecule containing the backsplice junction sequence. However, a single pre-mRNA can generate multiple circRNAs containing the same backsplice junction but altered exon/intron combination due to alternative splicing occurring within the circRNA during backsplicing. Additionally, the sequence of the circRNAs matches with the counterpart linear RNA, making it difficult to identify the mature spliced sequence of circRNA from RNA-seq data. To overcome this issue, we also describe the circRNA-rolling circle amplification (circRNA-RCA) method that enables the identification of full-length sequence of the circRNAs by Sanger sequencing after PCR amplification [17]. Since the function of circRNA depends on its sequence, identification of actual spliced sequence is a crucial step for predicting their biological function. Together, this chapter discusses two important PCR methods to validate the backsplice junction and full-length spliced sequence of circRNAs, which will accelerate the functional characterization of circRNAs.

2 Materials

2.1 Divergent and Full-Length Primer Design

1. Desktop system with web browsers such as Google Chrome or Mozilla Firefox.
2. Retrieving mature circRNA sequences from CircBase, CircInteractome, UCSC browsers, etc.
3. Divergent primer design with NCBI Primer-BLAST, Primer3, and CircInteractome web tool.

2.2 RNase R Treatment and RT-qPCR

1. 1.5 ml microcentifuge tube.
2. 0.2 ml PCR tubes.
3. PureLink RNA mini Kit (Thermo Fisher Scientific).
4. Total RNA purified from cells.
5. RNase R (20 U/μl; Lucigen).
6. Random hexamer (50 μM; Thermo Fisher Scientific).
7. 100 mM dNTPs (dATP, dCTP, dGTP, dTTP).
8. Ribolock RNase inhibitor (40 U/μl; Thermo Fisher Scientific).
9. Maxima H-minus reverse transcriptase (200 U/μl; Thermo Fisher Scientific).
10. Maxima reverse transcriptase (200 U/μl; Thermo Fisher Scientific).
11. Nuclease-free water.
12. Primer stocks (100 μM).
13. 2× PowerUp SYBR® Green master mix (Thermo Fisher Scientific).

2.3 Purification and Sanger Sequencing of PCR Products Amplified with Divergent and Full-Length Primers

1. Nuclease-free water.
2. Primer stocks (100 μM).
3. 0.2 ml PCR tubes.
4. 2× DreamTaq PCR master mix (Thermo Fisher Scientific).
5. DNA loading dye.
6. 1 kb Plus DNA Ladder (Thermo Fisher Scientific).
7. PureLink Quick Gel Extraction Kit (Thermo Fisher Scientific).
8. 10× TBE Buffer.
9. Agarose (HiMedia).
10. SYBR Gold gel stain (Thermo Fisher Scientific).
11. HiPurA PCR purification kit (HiMedia).

3 Method

All reagents should be nuclease free. Wear gloves all the time and prepare all reactions in a PCR workstation to avoid RNase-contamination during the assay.

3.1 CircRNA PCR with Divergent Primers

3.1.1 Designing Divergent Primers

1. Get the mature spliced sequence for the circRNA of interest from RNA-seq data or from any circRNA database with the circRNA sequence. Mature sequence of circRNA can be retrieved from the UCSC genome browser (https://genome.ucsc.edu/) by joining all the exon sequences present between the backsplice site genomic coordinates (*see* **Note 1**).

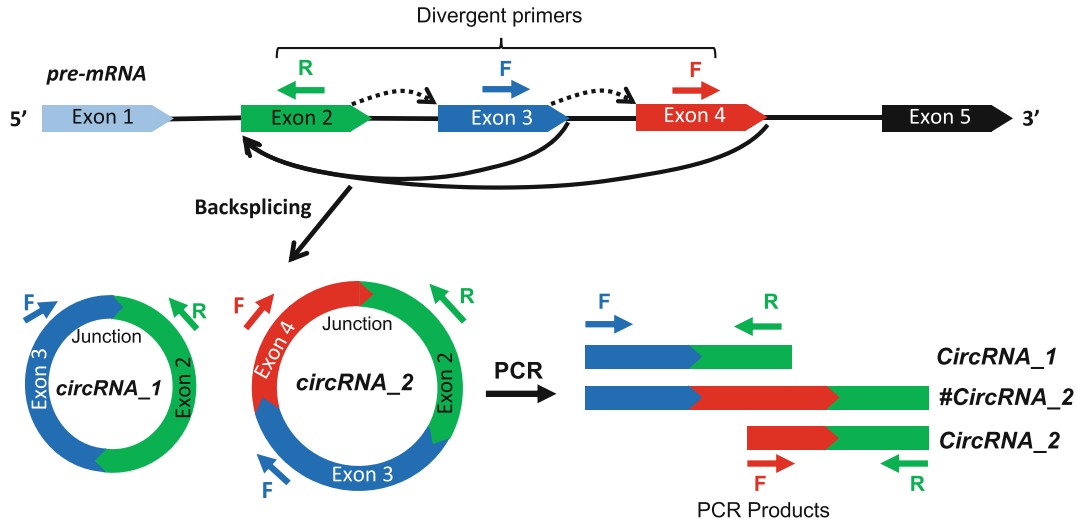

Fig. 1 Schematic representation of backsplicing generating circRNA from the pre-mRNA (*top*). Schematic showing the design of forward (F) and reverse (R) divergent primers used for validation and quantification of circRNAs (*bottom*). Divergent primer pair targeting one backsplice site may amplify multiple circRNAs coming from the same gene. Hashtag (#) represents unintended amplification of *circRNA_2* with divergent primers targeting *circRNA_1*

2. Make a circRNA-PCR amplicon template of length 200 nt including the backsplice junction sequence by joining the last 100 nt from the 3′ end sequence to the 5′ end of the first 100 nt.
3. CircRNA with a length of less than 200 nt can be divided into two halves. Add the 5′ half at the end of the 3′ half to prepare the backsplice junction PCR template for designing divergent primers.
4. Use NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) [18] web tool to design the PCR primers for this 200 nucleotides -long circRNA junction sequence template with selecting the PCR product size ranging from 120 to 160 nucleotides (Fig. 1) (*see* **Note 2**).
5. Design primers with length ~ 20 bp and with T_m within 58–60 °C.
6. Divergent primers for human circRNAs with circBase IDs can be designed with the circInteractome web tool (https://circinteractome.nia.nih.gov/) [19].

3.1.2 RNase R Treatment and cDNA Synthesis

1. For RNase R digestion, prepare a 20 µl reaction containing 2–5 µg of total RNA, 2 µl of 10× RNase R buffer, 1 µl of RNase R, 0.5 µl of Ribolock, and adjust the volume with nuclease-free water. Prepare a control reaction without RNase R enzyme (*see* **Note 3**).

2. Mix the reaction by tapping and centrifuge for a few seconds to settle the reaction at the bottom of the tube
3. Incubate the control and RNase R reaction tubes at 37 °C for 30 min.
4. Purify the control and RNase R treated RNAs using PureLink RNA isolation kit and elute in 40 μl nuclease-free water.
5. Take 1 μg of total RNA or equal volume of control and RNase R treated RNA in a 1.5 ml microcentrifuge tube for cDNA synthesis (*see* **Note 4**).
6. Prepare a 20 μl cDNA synthesis reaction by adding 1 μl of random hexamer (50 μM), 0.5 μl of Ribolock (40 U/μl), 1 μl of dNTP mix (10 mM of dATP, dTTP, dGTP, and dCTP), 1 μl of Maxima reverse transcriptase enzyme (200 U/μl), 4 μl of 5× Maxima RT buffer to the above RNA and adjust the volume with nuclease-free water (*see* **Note 5**).
7. Mix the reaction by tapping a few times, followed by a short spin to bring the reaction to the tube's bottom.
8. Incubate the cDNA synthesis reaction for 10 min at 25 °C followed by 60 min at 50 °C (*see* **Notes 4** and **6**).
9. Incubate the reaction at 85 °C for 5 min to inactivate the RT enzyme, followed by a quick chill on ice.
10. Add 500 μl of nuclease-free water to dilute the cDNA (*see* **Note 7**).
11. Use the prepared cDNA in the PCR or store at −20 °C till further use.

3.1.3 Identification of circRNA by PCR with Divergent Primer

1. Thaw the cDNA, 2× DreamTaq PCR master mix, and 100 μM primer stock at room temperature.
2. Vortex all solutions for a few seconds and keep on ice.
3. Take 10 μl of forward and reverse primer from the 100 μM stock solution and add 980 μl of nuclease-free water to prepare the 1 μM working solution of divergent primer mix. Vortex the primer mix thoroughly, followed by a short spin.
4. Prepare a 20 μl PCR reaction containing 10 μl of 2× DreamTaq PCR master mix, 5 μl of diluted cDNA, and 5 μl of 1 μM divergent primer mix. Vortex the PCR tube followed by short spin (*see* **Note 8**).
5. Setup the PCR on a thermal cycler with the initial step of 95 °C for 2 min followed by 40 cycles of 95 °C for 2 s and 60 °C for 5 s (*see* **Note 9**).
6. Store the PCR product at −20 °C or use it immediately for agarose gel analysis.
7. Prepare a 2% agarose gel in 1× TBE supplemented with 1× SYBR Gold.

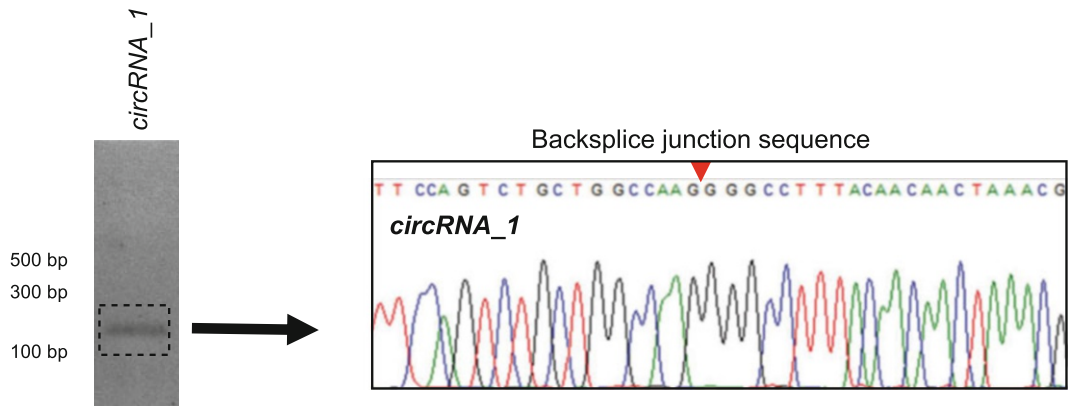

Fig. 2 Example circRNA backsplice junction sequence PCR amplified with divergent primers and visualized on 2% agarose gel stained with 1X SYBR Gold (*left*). Representative Sanger sequencing data of the circRNA PCR product showing the backsplice junction sequence (*right*). The red arrowhead represents the circRNA backsplice junction

8. Resolve the PCR product on the above agarose gel followed by an analysis of the PCR product's length on a transilluminator. The PCR reaction should show a single product of expected size (Fig. 2) (*see* **Notes 9** and **10**).
9. Purify the PCR product using the PCR purification kit or Gel extraction kit following the manufacturer's instructions (Fig. 2).
10. Confirm the amplification of circRNA backsplice junction sequence by Sanger sequencing using one of the divergent primers (Fig. 2).

3.1.4 Validation of Circularity by Quantitative (Q)PCR

1. Thaw the cDNA prepared from control and RNase R treated RNA, PowerUp SYBR Green master mix (2×), and working solution of 1 μM divergent primer mix.
2. Set up the quantitative real-time PCR of 20 μl in a 96-well plate using 5 μl of cDNA, 5 μl of 1 μM divergent primer mix, and 10 μl of 2× PowerUp SYBR Green master mix (*see* **Note 11**).
3. Seal the plate and vortex for a few seconds to mix the reaction evenly, followed by a short spin to settle the reaction at the bottom of the well.
4. Set up the RT-qPCR on the QuantStudio 6 real-time PCR machine with an initial step of 95 °C for 2 min followed by 40 cycles of 95 °C for 2 s and 60 °C for 5 s. (*see* **Notes 9** and **12**).
5. Check the relative expression levels of linear RNAs (*GAPDH* or *ACTB* mRNA), and circRNAs of interest in the RNase R treated sample compared with the control treatment sample using the comparative delta-CT method [20] (*see* **Note 13**).

3.2 CircRNA PCR with Full-Length Primers

The PCR with divergent primers can only validate the backsplice junction sequence while the mature circRNA sequence is derived from the transcriptomic data computationally. However, multiexonic circRNAs can have multiple circRNA splice variants containing a different combination of exons/introns with the same backsplice junction. Here, we provide the detailed protocol to design full-length PCR primers and perform circRNA-RCA that identifies the actual mature sequence of circRNA and its alternatively spliced isoforms.

3.2.1 Full-Length circRNA Primer Design

1. Get the mature sequence for the circRNA of interest from UCSC genome browser or RNA-seq data or any of the circRNA databases including CircNet, CircInteractome, etc. (*see* **Note 1**).
2. Take the 10 nt from the 3′ end of the mature spliced circRNA sequence and add it to the 5′ end.
3. The first 20 nucleotides of the 5′ end of the above sequence containing the circRNA junction will be the full-length forward (fl-F) primer. Make the reverse complementary sequence of the last 20 nucleotides from the 3’ end of the mature sequence and consider that as the full-length reverse (fl-R) primer (Fig. 3).
4. Although the primers’ optimal length is around 20 nt, the length can vary to have the primer Tm between 58–60 °C.

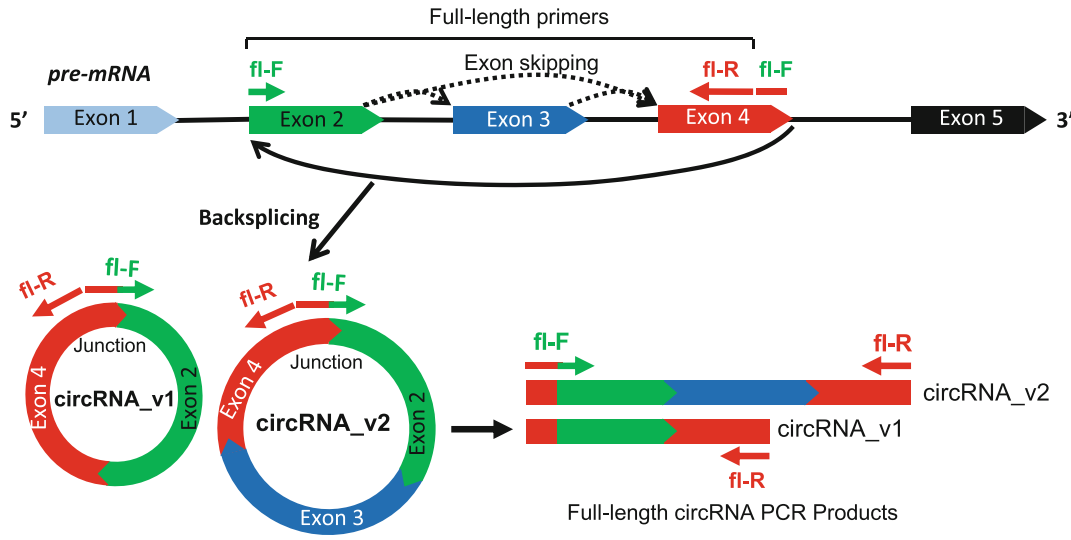

Fig. 3 Schematic representation of the biogenesis of two circRNA splice variants with same backsplice site and different exon combinations (*top*). The full-length forward primer is placed on the backsplice site while the full-length reverse primer is placed upstream of the forward primer. PCR with full-length PCR primers can amplify circRNA splice variants with different internal sequences (*bottom*)

3.2.2 Rolling Circle Reverse Transcription to Generate Full-Length cDNA

1. Take equal volume of RNA from control and RNase R treated sample in a 1.5 ml microcentrifuge tube for full-length cDNA synthesis.
2. Prepare a 20 μl cDNA synthesis reaction by adding 1 μl of random hexamer (50 μM), 0.5 μl of Ribolock, 1 μl of dNTP mix (10 mM), 4 μl of 5× Maxima reverse transcriptase buffer, and 1 μl of Maxima H minus reverse transcriptase enzyme to the above RNA (*see* **Note 14**).
3. Mix the reaction thoroughly by tapping the tubes a few times, followed by a short spin.
4. Incubate the reaction for 10 min at 25 °C followed by 60 min at 50 °C for cDNA synthesis (*see* **Note 6**).
5. Add 1 μl of RNase H and incubate cDNA reaction for 15 min at 37 °C.
6. Incubate the reaction at 85 °C for 5 min to inactivate the reverse transcriptase, followed by a quick chill on ice.
7. Add 500 μl of nuclease-free water to dilute the full-length cDNA stock for immediate use or store at −20 °C (*see* **Note 7**).

3.2.3 CircRNA PCR Using Full-Length Primers

1. Thaw the full-length cDNA, 2× DreamTaq PCR master mix, and full-length forward and reverse primer stock (100 μM) solutions at room temperature.
2. Prepare the 1 μM full-length primer mix by adding 10 μl of the fl-F and fl-R primers from the 100 μM stock solution to 980 μl of nuclease-free water. Vortex the primer mix thoroughly, followed by a short spin.
3. Prepare a 20 μl PCR reaction in a PCR tube using 5 μl of full-length cDNA prepared with RNase H-minus RT, 5 μl of 1 μM full-length primer mix, and 10 μl of 2× DreamTaq PCR master mix.
4. Seal the PCR tube and vortex it for a few seconds to thoroughly mix the reaction, followed by a short spin.
5. Set up the full-length circRNA PCR in a thermal cycler with the following cycling conditions: 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 58 °C for 20 s, and 72 °C for 60 s (*see* **Note 15**).
6. Store the PCR products at −20 °C or use them immediately for agarose gel analysis.

3.2.4 Identification of Full-Length Spliced Sequence of circRNA

1. Prepare a 2% agarose gel containing 1× SYBR Gold and 1× TBE buffer (*see* **Notes 16** and **17**).
2. Mix the full-length PCR product with DNA loading dye and resolve on an agarose gel along with DNA ladder.

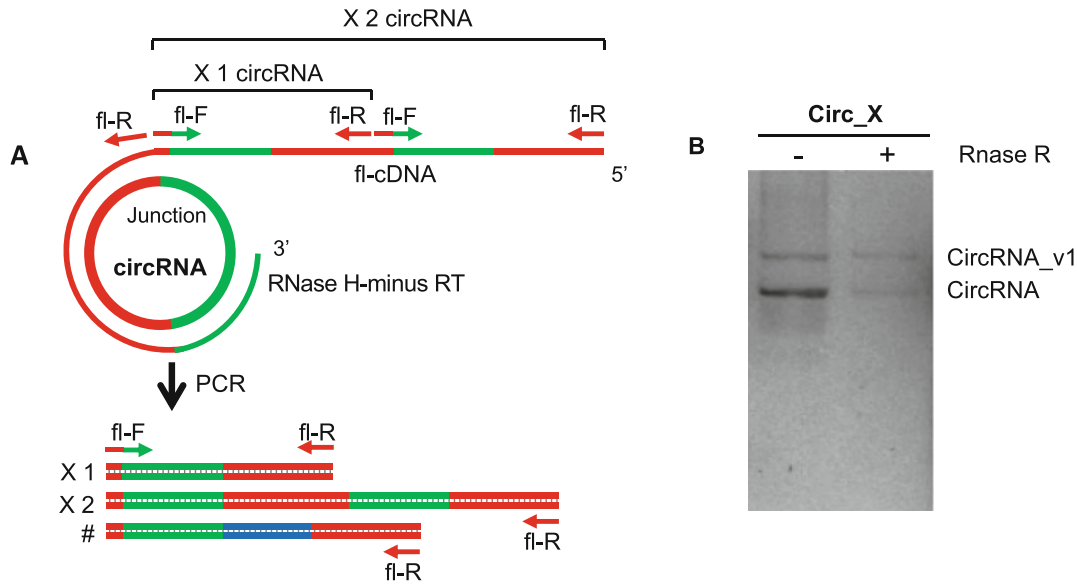

Fig. 4 circRNA PCR using full-length primers. (**a**) Schematic representation of rolling circle cDNA synthesis of circRNA with H minus reverse transcriptase. PCR amplification of specific circRNA using the above cDNA and full-length primers results in amplification of full-length circRNA (X 1) or doublet (X 2) of the target circRNA. Hashtag represents the amplification of circRNA splice variant. (**b**) Example full-length PCR products amplified using fl-F and fl-R primers were resolved on a 2% agarose gels stained with SYBR Gold. Two PCR products represent circRNA splice variants with same backsplice junction sequence

3. Visualize the gel on a transilluminator to analyze the size of the amplified PCR products for specific circRNA and their splice variants.
4. Amplification of multiple PCR products other than the expected circRNA size warrants further sequencing of each amplified product (*see* **Note 18**).
5. As shown in Fig. 4, purify both the gel bands using the PureLink Quick gel extraction kit following the manufacturer's instruction (*see* **Note 19**).
6. Quantify the PCR products isolated from the gel (*see* **Note 20**).
7. Sequence each of the PCR products with the full-length forward or reverse primer using the Sanger sequencing protocol.
8. Sanger sequencing will identify the altered use of exon/intron in the circRNA splice variants during backsplicing.

4 Notes

1. The circRNA spliced sequence is predicted with the assumption that all the exons in between the backsplice site are included in the circRNA. Please consider the strand information when the sequences are obtained from the genome. Please

remember that multiexonic circRNAs can have different isoforms depending on the exons and/or introns included during circRNA biogenesis.

2. Divergent primers should not overlap the backsplice junction site.
3. RNase R treatment is not recommended for quantitation of circRNA expression by RT-qPCR. However, to examine the circularity of the target backsplice junction, RNase R treatment followed by qPCR must be performed. Additionally, preheating the RNA at 70 °C for 5 min before treating it with RNase R may improve the degradation of linear RNAs with secondary structures.
4. The reverse transcription reaction can also be prepared in a PCR tube or PCR plate. For PCR tubes/plates, perform the reverse transcription reaction on a thermal cycler with a heated lid at 105 °C.
5. Any reverse transcriptase or cDNA synthesis kit can be used to prepare cDNA for circRNA analysis. Since circRNAs lack poly-A tail, the cDNA must be prepared with random hexamer only.
6. Incubation at 50 °C may cause evaporation of the reaction in the tube. The reaction tube may be centrifuged for a few seconds to bring the reaction to the bottom of the tube after 60 min of incubation at 50 °C.
7. The volume of water to dissolve the prepared cDNA depends on the initial amount of RNA taken for cDNA synthesis and the abundance of the target circRNA.
8. In this protocol, we used DreamTaq DNA polymerase as an example. Any Taq polymerase or PCR master mix can be used for PCR.
9. The time given for each PCR step is for reference only. Those can be modified depending on the PCR amplicon size and Taq polymerase used in the PCR. However, the duration of the PCR extension step is crucial for specific amplification of target circRNA. It is highly recommended to use two-step PCR or qPCR with a combined annealing and extension time of 5 s which allows specific amplification of the target circRNA backsplice junction without the amplification of other circRNAs. As shown in Fig. 1, higher annealing and extension time may allow nonspecific amplification of other circRNAs generated from the same gene.
10. Since most circRNAs are less abundant than the linear mRNAs, it is highly recommended to include a No-RT and water control in the PCR.
11. Any SYBR Green master mix can be used for qPCR.

12. Since the same gene can generate multiple circRNAs, the amplification of a specific circRNA for the first time in PCR and RT-qPCR must be validated by gel electrophoresis and melt curve analysis. Any primer set amplifying more than one product should not be used for RT-qPCR. RT-qPCR reactions should be performed in three biological replicates, with two technical replicates for each biological replicate.
13. The linear RNAs are expected to be degraded by RNase R treatment without affecting the circRNAs due to lack of free 3′ end.
14. Other RNase H-minus reverse transcriptase can be used in place of RNase H-minus Maxima RT enzyme for full-length cDNA synthesis.
15. The extension time for the full-length PCR can be changed depending on the length of the circRNA spliced sequence and the Taq polymerase used for PCR. We recommend an extension time of 1 min per kb of PCR amplicon.
16. Agarose gels can be stained with ethidium bromide or SYBR safe or any other DNA staining dye.
17. The percentage of the gel can be changed depending on the length of the circRNA.
18. All the PCR products amplified using full-length primers must be sequence-verified to find the actual sequence of the circRNAs. Multiple bands in circRNA-RCA do not always mean splice variants. The synthesis of long tandem repeats of full-length cDNA with RNase H-minus RT may result in multiple rounds of circRNA amplification.
19. Preheating the elution buffer or nuclease-free water at 65 °C may increase the yield of purified PCR product.
20. If the PCR product concentration is too low for Sanger sequencing, the specific band can be PCR amplified again with the full-length primers followed by sequencing of the purified PCR product.

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Conflicts of interest: The authors declare no conflict of interest.

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