Chapter 7

RT-qPCR Detection of Senescence-Associated Circular RNAs

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Abstract

Primary cells that reach the end of their replicative potential, encounter sublethal stress, or experience the activation of certain oncogenes cease proliferation and enter a state of long-term growth arrest named senescence. The senescent process has been implicated in a variety of age-related diseases and also in the pathogenesis of cancer. Senescence is characterized by distinct changes in the types and levels of coding RNAs (mRNAs) as well as in the vast collective of regulatory noncoding (nc)RNAs, which includes microRNAs, long noncoding RNAs (lncRNAs), and circular (circRNAs). Numerous technologies permit the detection of senescence-associated linear transcripts (mRNAs, lncRNAs, microRNAs), but the identification and quantification of circRNAs in senescence require distinct molecular approaches. In this chapter, we describe a method for the detection and measurement of circRNAs in senescent cells using specialized reverse transcription (RT) followed by real-time quantitative (q)PCR analysis.

Key words RNA-binding proteins, Sponge circRNAs, Divergent primer design, Transcriptome

1 Introduction

Circular RNAs (circRNAs) have been identified in numerous species, including human, mouse, nematode, and coelacanth. They are believed to function as regulators of gene expression at least in part by sponging microRNAs (miRNAs) [1–3]. One of the mechanisms through which circRNAs modulate gene expression is by functioning as "sponges" of miRNAs, sequestering miRNAs, and diminishing their availability to reduce the stability or translation of target mRNAs [3]. According to other proposed molecular functions, circRNAs may also serve as sponges for RNA-binding proteins (RBPs), provide platforms for assembly of RBPs, and associate with mRNAs to modulate their expression posttranscriptionally [4].

To assess the expression of circRNAs as a function of senescence, we used early-passage, proliferating (P) [population doubling (PDL) 20] and nonproliferating senescent (S) (PDL 52) cells. We isolated total RNA from these cells using TRIzol, enriched

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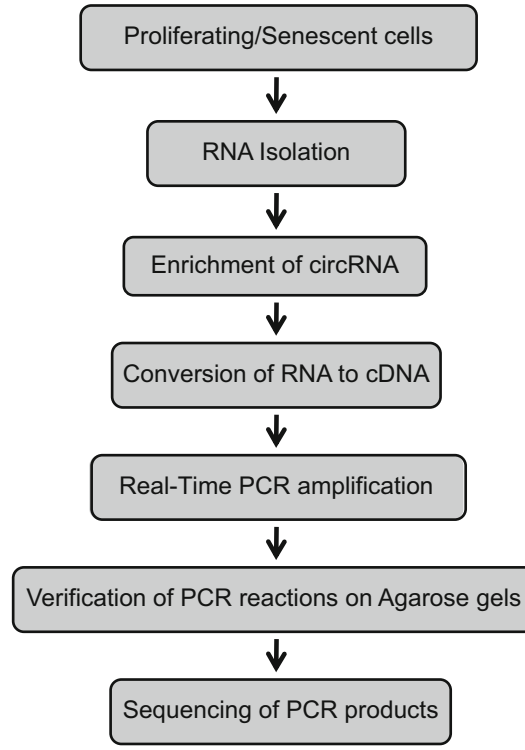

Fig. 1 Flowchart of circRNA quantification by RT-qPCR analysis in proliferating and senescent cells. After isolating total RNA, circRNAs are enriched by digestion with RNase R, converted to cDNA by RT, and quantified by qPCR using divergent primers. The amplified qPCR product is then verified on agarose gels and by DNA sequencing. Differentially expressed circRNAs can then be compared between proliferating and senescence cells

the concentration of circRNAs by RNase R treatment, and identified circRNAs by reverse transcription (RT) followed by real-time, quantitative (q)PCR analysis. To assess the levels of circRNAs, we used divergent primers and verified the products on agarose gels and by DNA sequencing (*see* Fig. 1). The data obtained from RT-qPCR reactions were used to calculate the changes in circRNA expression between P and S cells.

2 Materials

Caution: all of the reagents, materials, and instruments should be handled with care that they remain nuclease-free.

2.1 RNA Isolation

1. Cultures of proliferating cells (P) and cells rendered senescent (S) by replicative exhaustion (e.g., WI-38 fibroblasts) [5] or by oncogene-induced senescence (OIS) [6].

2. Dulbecco's phosphate-buffered saline (DPBS).
3. Vortexer.
4. Cell scrapers.
5. TRIzol® reagent (stored in 4°C).
6. Nuclease-free water.
7. RNase-free 1.5 mL microcentrifuge tubes.
8. NanoDrop spectrophotometer.

2.2 CircRNA Enrichment Using RNase R (Optional, See Notes 1–3)

RNase R treatment is required to degrade linear RNAs in order to enrich for circRNAs in an RNA sample. It is particularly helpful for circRNA identification by sequencing and microarray analysis. However, detection of abundant circRNAs by RT-qPCR may not require RNase R treatment, since divergent primers do not amplify linear RNAs.

1. Total RNA isolated from proliferating and senescent cells (*see* RNA isolation).
2. RNase-free 1.5 mL microcentrifuge tubes.
3. 20 U/μL RNase R.
4. RNase R 10× reaction buffer [0.2 M Tris–HCl (pH 8.0), 1 M KCl, and 1 mM $MgCl_2$] (including enzyme).
5. 40 U/μL RiboLock RNase inhibitor.
6. Eppendorf® Thermomixer® R.
7. 5:1 acidic phenol–chloroform.
8. 3 M sodium acetate solution, pH 5.2.
9. 15 mg/mL GlycoBlue™ coprecipitant.
10. NanoDrop spectrophotometer.

2.3 cDNA Synthesis by Reverse Transcription

Complementary DNA (cDNA) synthesis either from total RNA or RNase R-treated RNA is similar [1, 2]. For first-strand cDNA synthesis, random hexamers are used for priming. As the circRNAs lack the poly(A) tail, oligo(dT) priming is not recommended for cDNA synthesis.

1. Total RNA prepared with or without RNase R treatment.
2. Nuclease-free water.
3. 150 ng/μL random primer sets.
4. 10 mM dNTP mix.
5. 20–40 U/μL RNase inhibitor (e. g., RNasin ribonuclease inhibitor).
6. Maxima Reverse Transcriptase.
7. 5× RT buffer (250 mM Tris–HCl (pH 8.3 at 25°C), 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT) (provided with Maxima Reverse Transcriptase).

2.4 Real-Time PCR: Amplification and Sequence Validation

Use SYBR Green for PCR amplification of circRNAs. Successful amplification of a given circRNA is evidenced by the amplification curve, the dissociation curve, and visualization of the qPCR products on agarose gels, as described below.

1. cDNA from Subheading 2.3.
2. Nuclease-free water.
3. Forward and reverse primers for a housekeeping gene. Choose one or several abundant RNAs that do not change with senescence such as *18S* rRNA and *GAPDH* mRNA, and design the primers using Primer 3 online tool (http://bioinfo.ut.ee/primer3-0.4.0/) or NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
4. Junction-spanning divergent primers specific for the circRNA of interest. These can be designed using our free online web tool CircInteractome (http://circinteractome.irp.nia.nih.gov/Divergent_Primers/divergent_primers.html) [7].
5. Used KAPA SYBR® FAST qPCR Kits or SYBR Green from other vendors.
6. MicroAmp® Optical 96-Well Reaction Plate.
7. MicroAmp® Optical Adhesive Film.
8. QIAquick Gel Extraction Kit (50).
9. MPS 1000 Mini Plate Spinner.
10. Real-time PCR machine.

3 Methods

3.1 RNA Isolation

1. Wash cells twice with DPBS (PBS can also be used instead).
2. Immediately afterwards, add 1 mL of TRIzol to the cell plate, incubate them at room temperature for 10 min with rocking, and harvest into RNase-free 1.5 mL microcentrifuge tubes.
3. Add 0.2 mL (1/5 vol of TRizol) of chloroform and vortex for 10 s at maximum speed.
4. Centrifuge the tubes at 15,000 × *g* for 10 min at 4°C.
5. Carefully transfer 200 μL of the top aqueous layer to a fresh 1.5 mL RNase-free microcentrifuge tube without disturbing the middle layer.
6. Immediately, add 200 μL of 2-isopropanol (equal volume as the aqueous phase) and mix it by inverting the tube for 5–10 times and incubate for 10 min at room temperature or 30 min on ice.
7. Spin at 15,000 × *g* for 10 min at 4°C then remove the supernatant completely.

8. Add 1 mL of ice-cold 70% ethanol (70 mL ethanol mixed with 30 mL of nuclease-free water), vortex briefly, then centrifuge at 15,000 × *g* for 10 min at room temperature.
9. Remove the supernatant completely and air-dry the RNA pellet for 2–3 min at room temperature.
10. Dissolve the pellet by adding 50 μL of nuclease-free water, vortex to dissolve and place on ice. This RNA can be used directly or stored at −20 or −80°C for later use.
11. The RNA concentration and total amount of RNA isolated from each sample can be determined using the NanoDrop spectrophotometer. Ratio of absorbance at 260/280 in the range of 1.8–2.00 indicates good RNA quality.

3.2 CircRNA Enrichment Using RNase R (Optional)

1. Mix 5 μg total RNA with 2 μL RNase R 10× reaction buffer, 1 μL RNase R, and 1 μL RiboLock, and adjust the final volume to 20 μL using RNase-free water. Incubate at 37°C for 15 min.
2. Add 180 μL of RNase-free water and mix. Add 200 μL of acidic phenol–chloroform (5:1), vortex for 10 s and centrifuge at 15,000 × *g* for 5 min at room temperature.
3. Collect 150 μL from the aqueous top phase. Add 15 μL of sodium acetate (3 M, pH 5.2), 1 μL GlycoBlue and 375 μL (2.5 times the volume of the aqueous phase extracted) of 100% ethanol, and mix well by inverting the tubes for 5–10 times.
4. Incubate the tubes at −20°C for 1 h and centrifuge at 15,000 × *g* for 10 min at 4°C to precipitate the RNA.
5. Remove the supernatant carefully without disturbing the pellet. Add 1 mL of 70% ethanol and vortex the tube for a few seconds. Centrifuge at 15,000 × *g* for 10 min at room temperature, remove the supernatant completely, and air-dry the RNA pellet for 2–3 min at room temperature.
6. Add 20 μL of nuclease-free water and vortex until dissolved, then quickly place the dissolved RNA on ice or store until use.

   The RNA concentration can be determined using the NanoDrop spectrophotometer.

3.3 cDNA Synthesis by Reverse Transcription

1. Each RNA sample was used for two reactions: with reverse transcription (RT) and without RT.
2. For "with RT" reactions, use 1 μg RNA, 1 μL random hexamers, 4 μL 5× RT buffer, 1 μL RiboLock, 1 μL dNTP mix, 1 μL Maxima RT, and RNase-free water to 20 μL final volume. For "without RT" reactions, leave Maxima RT out: 1 μg RNA, 1 μL random hexamers, 4 μL 5× RT buffer, 1 μL RiboLock, 1 μL dNTP mix, and RNase-free water to a final volume of 20 μL.

3. Mix and spin for a few seconds to settle the reaction mixture at the bottom of the tube.
4. Incubate at room temperature (25°C) for 10 min followed by 30 min incubation at 50°C using a Thermomixer.
5. Inactivate the RT enzyme by heating the reaction at 85°C for 5 min.
6. Dilute the reaction to 500 μL with 480 μL of nuclease-free water.
7. The cDNA product can be stored at −20 or −80°C or used immediately for qPCR amplification.

3.4 Real-Time PCR: Amplification and Sequence Validation

3.4.1 Preparation of qPCR Primers

1. Dissolve primers (*see* Fig. 2) using RNase-free water to a final concentration of 100 μM.
2. Prepare the primer mix of forward and reverse primers at a final concentration of 1 μM, each in nuclease-free water. For example, add 10 μL of each primer from the 100 μM stock into 980 μL of nuclease-free water and mix well.

3.4.2 Quantitative PCR (qPCR): (See Note 4)

1. Cover the wells with optical adhesive film, and mix the reaction by vortexing.
2. Spin the 96-well plate for 30 s using MPS 1000 Mini Plate Spinner to settle the reactions at the bottom of the wells.
3. Set up the ABI 7500 qPCR machine as follows: 5 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. It is recommended to add dissociation curve analyses at least when testing new primers.

3.4.3 Validation of PCR Product

1. The PCR amplification curves should include a linear portion (*see* Fig. 3a). A single peak in the dissociation curve represents one PCR product from the amplification reaction (*see* Fig. 3b). To validate the PCR product, it can be resolved on a 2% agarose gel containing ethidium bromide. The "no RT" reaction should not show any PCR product, while the RT reaction should show a single DNA product matching the expected size of the PCR amplicon (*see* Fig. 3c). The expected PCR product can be purified from the agarose gel by using the QIAquick Gel Extraction Kit following the manufacturer's protocol. To verify the PCR product by sequencing, forward or reverse primer can be used for the PCR sequencing, which should reveal the junction sequence of the circRNA (*see* Fig. 3d). Once the circRNA PCR amplification is validated, one can proceed for data analysis to find out the change in that particular circRNA during senescent compared to proliferating cells.

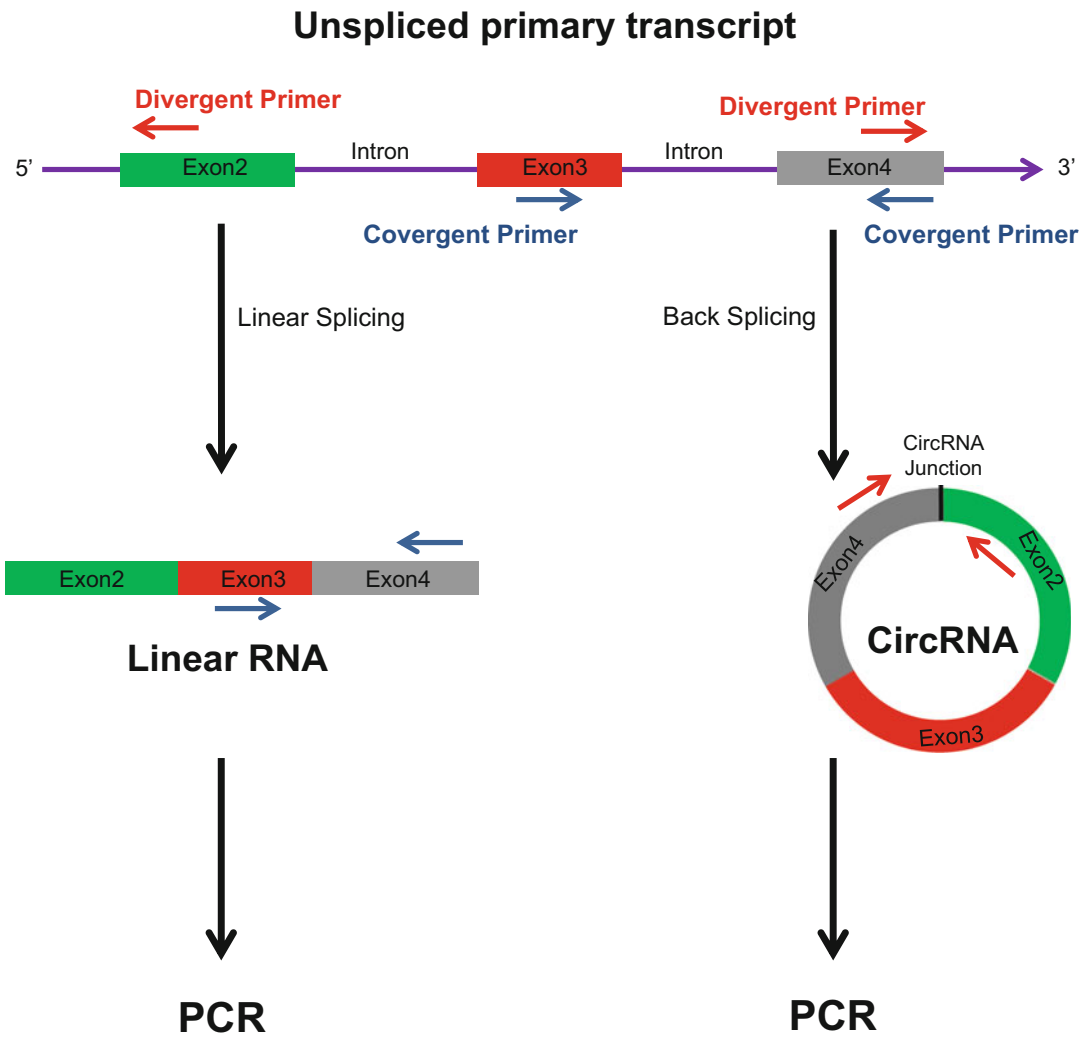

Fig. 2 Schematic depiction of linear RNA and circRNA from the same original primary transcript. Schematic illustration of the linear mature RNA generated from linear splicing (*left*) and the circRNA generated from back splicing (*right*) of the primary RNA. Convergent and divergent primers are used to amplify the linear and circular RNAs, respectively

3.4.4 Data Analysis

1. As shown in Fig. 3a, the threshold is positioned in the linear part of exponential amplification phase. The cycle threshold (Ct) is the number of cycles required for the fluorescent signal to cross the threshold. The Ct values are used to calculate the difference in circRNA expression between proliferating and senescent cells. The Ct values for each primer set obtained from proliferating cells were compared to those seen when amplifying cDNA from senescent cells. The differences between the Ct values for housekeeping transcripts

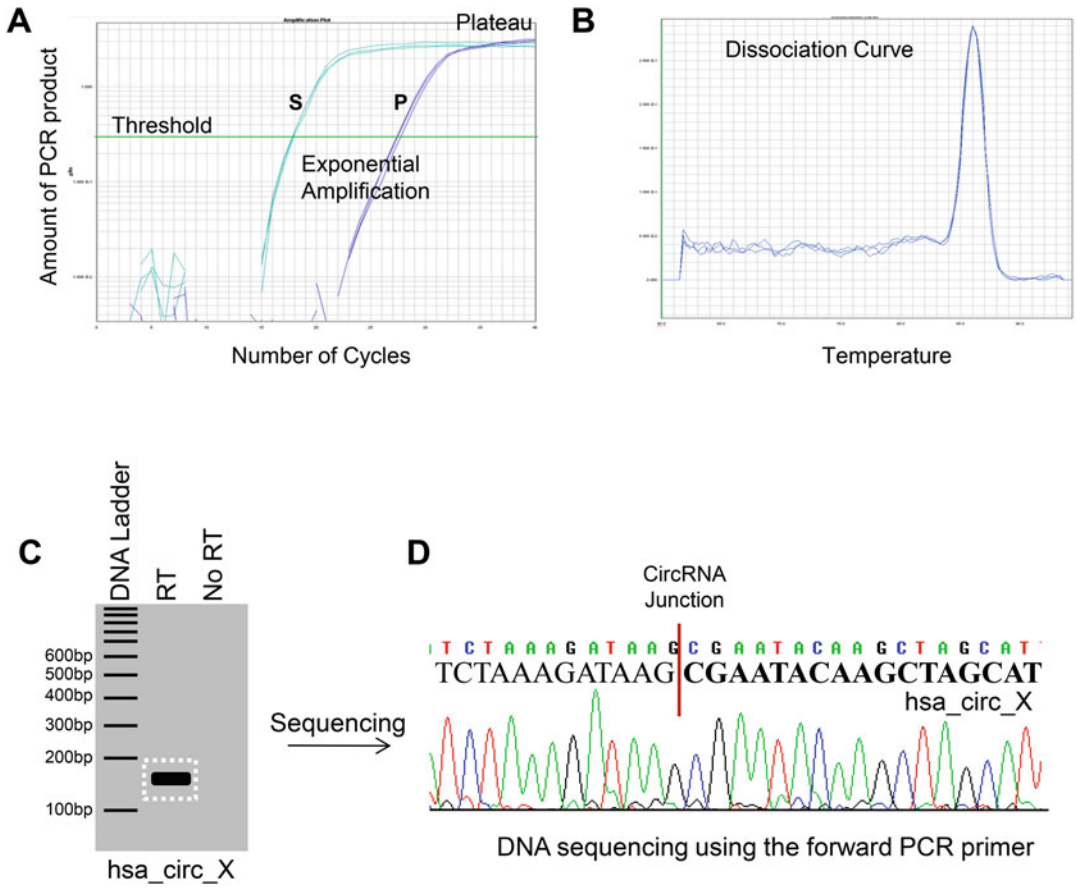

Fig. 3 Amplification and validation of circRNA. (**a**) Logarithmic amplification plot of RT-qPCR reactions; Ct values determine where the threshold line crosses the plot. (**b**) A single-peak melting curve indicates that a single major PCR product was amplified. (**c**) Schematic illustration of RT-qPCR products visualized by electrophoresis in ethidium bromide-stained agarose gels. (**d**) DNA sequencing analysis of the PCR product from panel **c** using the forward primer to confirm the circRNA junction sequence

are used to normalize the Ct values for each circRNA. The fold change in expression of any circRNA in senescent cells compared to proliferating cells is calculated using the formula $2^{\Delta CT\ (Ct\ young\text{-}Ct\ senescence)}$ (*see* ref. 8) (*see* **Note 5**).

4 Notes

1. All of the reagents, materials, and instruments should be handled with care that they remain nuclease-free. Thaw the reagents on ice. Before the incubation, mix the reaction components by tapping the tube, and spin briefly (10,000 × *g* for 5 s) to pull the contents to the bottom of the tubes.
2. This RNA contains enriched circRNAs which can be directly used for cDNA synthesis.

3. Unit definition: 1 U of RNase R can degrade 1 μg of poly(A)+linear RNA into acid-soluble nucleotides in 10 min at 37°C under standard assay conditions.
4. For one reaction in 96-well plate, add 10 μL SYBR Green, 5 μL cDNA, and 5 μL primer mix. The same mixture can be prepared for "no RT" reaction. For a large number of reactions, a master mix can be prepared.
5. Example: For circRNA-X, the Ct for proliferating cells is 27.8, and the Ct for senescent cells is 17.8. The fold change in circRNA is $X = 2^{(27.8\text{-}17.8)} = 2^{10} = 1024$. Thus, circRNA-X is upregulated 1024 fold in senescent cells compared to proliferating cells.

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