[*This is a protocol for inclusion in CPMB. There are 4 figures, 1 Table, and 2 Supplementary Tables]

Validation of circular RNAs using RT-qPCR after effective removal of linear RNAs by ribonuclease R

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Abstract

Circular RNAs (circRNAs) are a class of endogenous noncoding RNAs that have been shown to play a role in normal development, homeostasis, and disease, including cancer. CircRNAs are formed through a process called back-splicing, which results in a covalently closed loop with a nonlinear back-spliced junction (BSJ). In general, circRNA BSJs are predicted in RNA sequencing data using one of numerous circRNA detection algorithms. Selected circRNAs are then typically validated using an orthogonal method such as reverse transcription quantitative polymerase chain reaction (RT-qPCR) with circRNA-specific primers. However, linear transcripts originating from endogenous trans-splicing can lead to false-positive signals both in RNA sequencing and in RT-qPCR experiments. Therefore, it is essential to perform the RT-qPCR validation step only after linear RNAs have been degraded using an exonuclease such as ribonuclease R (RNase R). Several RNase R protocols are available for circRNA detection using RNA sequencing or RT-qPCR. These protocols, which vary in enzyme concentration, RNA input amount, incubation times, and cleanup steps, typically lack a detailed validated standard protocol and fail to provide a range of conditions that deliver accurate results. As such, some protocols use RNase R concentrations that are too high, resulting in partial degradation of the target circRNAs. Here, we describe an optimized workflow for circRNA validation, combining

RNase R treatment and RT-qPCR. First, we outline the steps for circRNA primer design ad qPCR assay validation. Then, we describe RNase R treatment of total RNA and, importantly, a subsequent essential buffer cleanup step. Lastly, we outline the steps to perform the RT-qPCR and discuss the downstream data analyses.

Basic Protocol 1: CircRNA primer design and qPCR assay validation
Basic Protocol 2: RNase R treatment, cleanup, and RT-qPCR

Keywords
circular RNA, ribonuclease R, noncoding RNA, RT-qPCR, primer design

INTRODUCTION

Circular RNAs (circRNAs) are a class of endogenous noncoding RNAs that are widespread and abundant in a variety of organisms. Although a precise function and working mechanism are lacking for the majority of them, circRNAs have been proposed to function as microRNA and protein sponges, and as modulators of transcription and splicing. Some circRNAs are aberrantly expressed in pathological conditions such as cancer (Meng et al., 2017; Lux and Bullinger, 2018).

Originating from the same precursor as linear RNA transcripts, circRNAs are formed through a process called back-splicing, which is a noncanonical form of alternative splicing. Back-splicing results in a covalently closed loop characterized by a nonlinear back-spliced junction (BSJ) between a splice donor and an upstream splice acceptor (Figure 1A) (Salzman et al., 2012). As such, circRNAs lack a poly(A) tail and 5' and 3' ends. The detection or quantification of circRNAs is based on the presence of their characteristic BSJ.

[*Figure 1 near here]

In general, large-scale circRNA detection is performed based on RNA sequencing data, using one or more circRNA detection algorithms (Gao and Zhao, 2018; Hansen et al., 2015; Zeng et al., 2017; Jakobi and Dieterich, 2019). The existence of circRNAs of interest is then evaluated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Tsitsipatis and Gorospe, 2020). Of note, linear transcripts can lead to false-positive circRNA signals both in RNA sequencing and in RT-qPCR experiments due to endogenous trans-splicing, which results in an exon orientation that is identical to the sequence surrounding the circRNA BSJ (Pfafenrot and Preußer, 2019). Therefore, it is essential to perform a circRNA validation step using RNase R, an exonuclease that specifically degrades linear RNAs (Vincent and Deutscher, 2006; Jeck et al., 2013; Memczak et al., 2013). RNase R treatment will not degrade all linear RNA completely, but it may do so to a sufficient degree to help identifywhich transcripts are RNase R susceptible (and, therefore, linear) and which ones are RNase R resistant (and, therefore, circular). Various RNase R protocols have been published for circRNA detection using RNA sequencing and

RT-qPCR (Panda et al., 2017; Panda and Gorospe, 2018; Sekar et al., 2019). These protocols, however, vary in enzyme concentration, RNA input amount, incubation times, and cleanup steps, lack a detailed description of a validated standard protocol, and fail to provide a range of conditions that deliver accurate results. As such, some protocols use RNase R concentrations that are too high, resulting in partial degradation of target circRNAs, affecting subsequent experiments.

Here, we describe a complete workflow for circRNA validation, including primer design, qPCR assay validation, RNase R treatment, buffer cleanup, and RT-qPCR. After selection of the circRNAs of interest, primers surrounding the circRNA BSJs are designed and validated using synthetic DNA template positive controls. Once adequate primers are identified, the total RNA sample is treated with RNase R to degrade linear RNAs. This RNase R treatment has been optimized using multiple conditions to ensure maximal reduction of linear RNA while maintaining the levels of circRNAs intact (see Background Information). After RNase R, an essential buffer cleanup step is performed using the Vivacon 500 Hydrosart 10 kDa columns (VN01H02, Sartorius Lab), which, in our hands, is more efficient and cost effective than similar approaches (see Background Information). Next, we show how the purified RNA can be used for RT-qPCR to assess the effect of the RNase R treatment and confirm the existence of the target circRNA through quantification of its BSJ. Here, we expect *bona fide* circRNAs to have similar abundance levels in both control and RNase R-treated samples. To illustrate this protocol, circRNAs expressed in 3 human cancer cell lines (SW480, NCI-H23, and HLF) were selected based on their high abundance in our in-house sequencing data (Table 1). Using this approach, we show how RNase R treatment followed by RT-qPCR is essential for the study and validation of circRNAs. Furthermore, although not the main subject of this protocol, the RNaseR treatment and ultrafiltration cleanup steps described here can also be used for validation of circRNAs using RNA sequencing instead of RT-qPCR.

[*Table 1 near here]

BASIC PROTOCOL 1: CIRCRNA PRIMER DESIGN AND QPCR ASSAY VALIDATION

To detect circRNAs using RT-qPCR, circRNA-specific primers need to be designed. These primers face away from each other when annealing to linear RNA (divergent orientation), preventing amplification, but will face towards each other (convergent orientation) when annealing to the circRNA, leading to intended amplification of the sequence encompassing the BSJ (Figure 1B). For the initial primer design, the Primer3Web tool (Untergasser et al., 2007) is used. Next, the most optimal primer pair is selected based on specificity, absence of SNPs, and absence of secondary structure in the amplicon.

Once designed, and before using these primers in a circRNA validation experiment, they need to be experimentally evaluated. To measure the PCR efficiency of a new primer pair, it is recommended that they are tested on a serial dilution series of a positive control RNA that contains the gene of interest (for example, Human Universal Reference RNA, Agilent #750500). However, as the circRNA of interest may not be expressed or be of low abundance in such a sample, synthetic DNA template positive controls can be used instead to validate the PCR efficiency of the primers. We describe the latter approach below.

Materials

- Primer3Web tool: https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
  (Untergasser et al., 2007)
- SsoAdvanced Universal SYBR Green Supermix (#172-5274, Bio-Rad)
- 10 ng/µl tRNA from Brewer's Yeast (Sigma, #10109517001) in nuclease-free water
- nuclease-free water
- 0.2 ml PCR tubes or strips
- thermal cycler
- qPCR plate
- qPCR instrument

Protocol Steps

Primer design

1. Retrieve the 300-nucleotide sequence encompassing the predicted BSJ of the circRNA of interest. Use the UCSC Genome Browser (Haeussler et al., 2019) to retrieve 150 nucleotides upstream of the predicted BSJ end position and concatenate to the 150 nucleotides downstream of the BSJ start position.
2. Paste the sequence in the Primer3Web tool (Untergasser et al., 2007) and generate candidate primers using the following settings: primer length (nucleotides) = 16-20-30 (min-opt-max); primer Tm: 58-59-60 (min-opt-max); max Tm difference: 2°C; primer GC %: 30-50-80 (min-opt-max); concentration of monovalent cations: 50 mM; concentration of divalent cations: 3 mM; amplicon product size: 60-250 nt.
3. Select a primer pair that satisfies the following criteria.
    a. Verify absence of secondary structure of the amplicon using UNAFold (Markham and Zuker, 2008). Use the following settings: annealing temperature of 60 °C; Mg^{++} and Na^{+} concentration depends on the qPCR mix (the suggested settings for a typical PCR supermix are 3 mM Mg^{++} and 50 mM Na+; check your mix).
    b. Verify the primers for off-target amplification using the ePCR tool from the BiSearch web tool (Arányi et al., 2006) by selecting the 'Homo sapiens cDNA' database with 123333333333333 as mismatch string.

As circRNAs are not present in the reference genome, this ePCR should not produce PCR products. Non-specific amplicons with at least 5 mismatches in the combined primer annealing regions or with at least 4 mismatches in one primer annealing region are unlikely to be amplified (Lefever et al., 2013); nevertheless, empirical validation of specificity is highly recommended (see also Troubleshooting).

c. Verify absence of frequent SNPs (> 1 % minor allele frequency) in the primer annealing regions, especially in the last 5 nucleotides (Lefever et al., 2013) using the UCSC 'Common SNPs' track (Haeussler et al., 2019).

4. Order the selected circRNA primers from any oligonucleotide synthesis provider (purification: standard desalting, amount: 25 nmol).

Primer validation on synthetic DNA template positive controls

5. Based on your primer design, determine the amplicon sequence (containing the circRNA BSJ). Concatenate the first 30 nucleotides and the last 30 nucleotides of the amplicon sequence to generate the 60-mer sequence of the synthetic DNA template positive control (Figure 1C) (see Supplementary Table 2 for sequences of the synthetic DNA template positive controls for the selected circRNAs used as an example here, for reference).

The synthetic template does not to include the circRNA BSJ. Such a template, however, is adequate to test the amplification efficiency of the primers.

6. Order the synthetic template (purification: standard desalting, amount: 25 nmol).

   Of note, make sure to order your primers a few days prior to ordering the synthetic template or order them from a different supplier. Otherwise, your primers may face the risk of being contaminated with template during synthesis. Also, treat the synthetic template as high-risk for cross-contamination and make the proper dilution in a post-PCR lab.
7. Make a synthetic template dilution series. The synthetic template is diluted with tRNA from Brewer's Yeast (carrier RNA) to prevent the synthetic template from sticking to the walls of the tubes and pipet tips (at low dilution).

a. Centrifuge the synthetic template 5 minutes at 10,000 g, room temperature.
b. Dilute the synthetic template to a working solution of 1,000,000 molecules/µl with 10 ng/µl tRNA.
c. Prepare a 6-point 10-fold serial dilution series of the synthetic template according to the table below. For point 1 (1,000,000 molecules/µl), use 200 µl of the working solution from b. For point 2, add 20 µl of point 1 to 180 µl of 10 ng/µl tRNA. Repeat this step serially for points 3-6, to create the dilution series. Vortex and spin-down the synthetic template between each dilution step.

	synthetic template		**10 ng/µl tRNA (µl)**	**final concentration (molecules/µl)**
point 1	200	µl work solution	0	1,000,000
point 2	20	µl point 1	180	100,000
point 3	20	µl point 2	180	10,000
point 4	20	µl point 3	180	1,000
point 5	20	µl point 4	180	100
point 6	20	µl point 5	180	10

8. Prepare two negative control samples: a negative control containing 10 ng/µl tRNA and a no-template control (NTC) containing nuclease-free water.
9. Vortex the 2x SsoAdvanced SYBR Green Supermix and spin down.
10. Vortex the forward and reverse primer well for at least 10 seconds and spin down.
11. Prepare a mastermix for each assay as described below. *In this example, the mastermix is calculated for 16 samples, with a 12-sample excess. The excess is to allow the mastermix to be distributed using a repetition*

pipet. If you are using a regular pipet, 10% excess should be sufficient (i.e. preparing a mastermix for 17.6 samples). Always try to avoid pipetting small volumes (<2 µl).

	volume (µl) for 1 sample	**volume (µl) for mastermix for 16 samples (+ 12 excess)**
2x SsoAdvanced SYBR Green Supermix	2.50	70.0
forward primer (5 µM)	0.25	7.0
reverse primer (5 µM)	0.25	7.0
total	3.00	84.0

12. Vortex each mastermix well for at least 10 seconds and spin down.
13. Distribute 3 µl of mastermix for each assay in the correct well of the 384-well plate (using a repetition pipet). Distribute 2 µl of the sample (and controls) in the correct wells using a multichannel pipet.

An example of a run lay-out using a 384-well qPCR plate is shown in Figure 2A: 4 circRNAs, 6-point dilutions series, including 2 negative controls (carrier RNA and NTC), with qPCR duplicates.

14. Seal the plate well and centrifuge it for 5 minutes at 400 g, room temperature.
15. Program the qPCR instrument as described below (here done for a LightCycler 480 II) and run the qPCR.

step		**temperature**	**time**	**ramp rate**	**acquisition mode**
pre-incubation		95 °C	2 min	4.8 °C/s	none
amplification	44 cycles	95 °C	5 sec	4.8 °C/s	none
		60 °C	30 sec	2.5 °C/s	none
		72 °C	1 sec	4.8 °C/s	single
melting curve		95 °C	5 sec	4.8 °C/s	none
		60 °C	1 min	2.5 °C/s	none

	95 °C		0.11 °C/s	continuous (5 per °C)
cooling	37 °C	3 min	2.5 °C/s	none

16. Analyze the qPCR data. The circRNA primers should only have one melting peak when using a double-stranded DNA binding dye (such as SYBR Green I), and this peak should be the same in all dilutions. The amplicon length can also be confirmed by electrophoresis of the amplicon. See Troubleshooting for more information. Primer efficiency should be between 90% and 110%, and can be calculated using qbase+ (Biogazelle, Zwijnaarde, Belgium; www.qbaseplus.com). Alternatively, primer efficiency can be calculated as described in (Hellemans et al., 2007).

[*Figure 2 near here]

BASIC PROTOCOL 2: RNASE R TREATMENT, CLEANUP, AND RT-QPCR

The validated circRNA primers described in Basic Protocol 1 can now be used for circRNA detection with RT-qPCR. However, as described in the introduction, qPCR with circRNA primers can also amplify endogenous trans-spliced linear transcripts, leading to false positive results. To prevent these, RNase R treatment of the RNA is performed prior to the RT-qPCR, which will make it possible to recognize true circRNAs based on the difference in Cq values between untreated and RNase R-treated samples. For this, total RNA is treated with RNase R to remove linear RNA. We highly recommend including a buffer-treated control (instead of using untreated RNA as baseline) and to perform each RNase R treatment *in duplo* (treatment replicates) to account for the variability of the treatment. Of note, it is recommended to include replicates as early as possible in any empirical workflow; as such, RNase R treatment replicates are more informative than RT replicates, which in turn, are more informative than qPCR replicates.

Subsequently, a buffer cleanup step is performed to remove residual inhibitory RNase R buffer from the sample. Finally, RT-qPCR is performed to measure the relative abundance of circular transcripts of interest. Additionally, assays to measure linear control genes (i.e. those whose transcripts undergo no known backsplicing and circularization, and which

should be degraded by the RNase R treatment) are included in the qPCR, to ensure that the RNase R treatment was successful. As an example, effective linear control assays for *ACTB*, *B2M*, *HPRT1* and *TBP* are described in Supplementary Table 1 (also previously described as candidate reference genes in (Vandesompele et al., 2002))

Materials

- RNA sample
- RNase R (#RNR07250, Epicentre)
- 10X RNase R Reaction Buffer: 0.2 M Tris-HCl (pH 8.0), 1 M KCl and 1 mM MgCl2 (provided with RNase R enzyme)
- Vivacon 500 Hydrosart 10kDa ultrafiltration columns (#VN01H02, Sartorius Lab)
- iScript Advanced cDNA Synthesis Kit (#172-5038, Bio-Rad)
- SsoAdvanced Universal SYBR Green Supermix (#172-5274, Bio-Rad)

- nuclease-free water
- 0.2 ml PCR tubes or strips
- thermal cycler
- qPCR plate
- qPCR instrument

- primers for circRNAs of interest (Basic Protocol 1) and linear control genes (Supplementary Table 1)

Protocol Steps

RNase R treatment

Here, as an example, we describe a protocol where 4 circRNAs are measured in one RNA sample, with treatment duplicates, resulting in two buffer-treated control samples and two RNase R-treated samples.

1. Dilute the RNA sample(s) to obtain 10-1000 ng total RNA in 10 µl of nuclease-free water in a 0.2 ml tube or strip. Pipet up and down at least 10 times and spin down. Keep the RNA on ice (or cool block).

Prepare one RNA sample for each condition. For this example, 40 µl of RNA (100 ng/µl) was divided over four tubes, two of which will be buffer-treated control samples and two will be RNase R-treated.

2. Spin down the RNase R enzyme. Dilute the RNase R enzyme (20 U/µl) with nuclease-free water to a working solution of 1 U/µl. Pipet up and down at least 10 times and spin down. Keep the enzyme on ice (or cool block). *This RNase R enzyme working solution can be stored at -20 °C for at least 5 months. It can presumably be stored even longer without loss of efficiency, but 5 months is the longest storage duration we have tested.*
3. Vortex the 10X RNase R Reaction Buffer and spin-down.
4. Spin down the RNase R enzyme working solution (1 U/µl, step 2). Keep the enzyme on ice (or cool block).
5. Prepare the mastermix for the control samples as described below. Keep the mastermix on ice (or cool block). Pipet the mastermix up and down at least 10 times and spin down.

We recommend always making a master mix for at least 2 samples, to avoid pipetting small volumes (<2 µl).

	volume (µl) for 1 sample	**volume (µl) for mastermix for 2 samples (+ 10 % excess)**
RNase R Reaction Buffer	1.5	3.3
nuclease-free water	3.5	7.7
total	5.0	11.0

6. Prepare the mastermix for the treated samples as described below. Keep the mastermix on ice (or cool block). Pipet the mastermix up and down at least 10 times and spin down.

We recommend always making a master mix for at least 2 samples, to avoid pipetting small volumes (<2 µl).

	volume (µl) for 1 sample	**volume (µl) for mastermix for 2 samples (+ 10 % excess)**
RNase R enzyme (1 U/µl)	1.0	2.2
RNase R Reaction Buffer	1.5	3.3
nuclease-free water	2.5	5.5
total	5.0	11.0

7. Add 5 µl of the control mastermix or of the RNase R mastermix to each corresponding RNA sample (Step 1). Pipet up and down at least 10 times and spin down.
8. Incubate for 15 min at 37 °C (if using a thermal cycler, set lid temperature to 40 °C). After incubation, keep the RNA samples on ice (or cool block).

Cleanup reaction

9. Prepare the Vivacon 500 Hydrosart 10 kDa ultrafiltration columns by inserting a concentrator body in a collection tube for each sample. Make sure the filter of the concentrator body is accessible and the concentrator body is not inverted.
10. Pipet 500 µl of nuclease-free water into the column.
11. Centrifuge for 9 minutes at 7500 g, room temperature.
12. Invert the concentrator body in the same collection tube and centrifuge for 2 minutes at 2500 g, room temperature, to remove residual water from the filter.
13. Invert the concentrator body in the same collection tube once more, so the filter is accessible again.
14. Pipet the RNA sample (step 8, ~15 µl) completely onto the filter.
15. Centrifuge for 9 minutes at 7500 g, room temperature.

16. *Optional*: if your experiment requires highly concentrated samples, pipet the droplet that remains on the filter onto the middle of the filter again. Centrifuge for 3 min at 7500 g, room temperature. This will result in a final volume of 2-3 µl, instead of the 4-6 µl when this optional step is omitted.
17. Invert the concentrator body into a new collection tube (the filter is now facing down and is not accessible anymore).
18. Centrifuge for 2 minutes at 2500 g, room temperature, to collect the sample from the column. Put the collection tubes on ice (or cool block).
19. Transfer the eluate to a new 0.2 ml tube. Keep the samples on ice (or cool block).
20. Measure the volume of each sample and add nuclease-free water to obtain your desired final volume (in this example, 15 µl to perform the RT reaction).

Reverse transcription

21. Vortex the iScript Advanced 5x Reaction Mix and spin down.
22. Spin down the iScript Advanced Reverse Transcriptase. Keep the enzyme on ice (or cool block).
23. Prepare the mastermix as described below.
    For this example, the mastermix is prepared for 4 samples (2 untreated buffer-control and 2 RNase R-treated samples). We recommend always making a master mix for at least 2 samples, to avoid pipetting small volumes (<2 µl).

	volume (µl) for 1 sample	**volume (µl) for mastermix for 4 samples (+ 10 % excess)**
iScript Advanced Reverse Transcriptase	1.0	4.4
iScript Advanced 5x Reaction Mix	4.0	17.6
total	5.0	22

24. Pipet the mastermix up and down at least 10 times and spin down. Keep the mastermix on ice (or cool block).
25. Add 5 µl of the mastermix to each RNA sample (step 20). Pipet up and down at least 10 times and spin down. Keep the RNA samples on ice (or cool block).
26. Program the automated thermal cycler as described below and run the reaction.

	temp	**lid temp**	**time**
reverse transcription	46 °C	105 °C	20 min
RT inactivation	96 °C	105 °C	1 min

After the RT step, the cDNA samples no longer need to be kept on ice and can be stored at -20 °C (safe stopping point).

qPCR

As the goal of this experiment is to compare circRNA expression levels among multiple samples (each with their RNase R-treated and buffer-treated versions), sample maximization is the preferred set-up. This means that as many samples as possible are analyzed in the same run, and that different genes should be analyzed in different runs if not enough free wells are available to analyze the different genes in the same run (Derveaux et al., 2010). If more targets need to be measured than fit in an actual run, then more runs should be included. Of note, it does not matter if reference genes or control genes are measured in a different plate (see also https://blog.qbaseplus.com/four-tips-for-rt-qpcr-data-normalization-using-reference-genes). If possible, include two qPCR replicates of each sample to control for pipetting variations (as mentioned above, it is recommended to include replicates as early as possible in any empirical workflow). Include an NTC for each assay. Include at least two linear control genes. An example of a run lay-out using a 384-well qPCR plate is shown in Figure 2B: one RNA sample was divided into buffer-treated control samples and RNase R-treated samples, each with treatment duplicates and qPCR duplicates. An NTC containing nuclease-free water is also

included. This results in a total of 5 samples, in which, for this example, 4 circRNAs and 2 linear control genes are measured.

27. Dilute the cDNA samples 1:4 by adding 60 µl of nuclease-free water to each sample. Pipet up and down at least 10 times to mix well and spin down.

Make sure that your cDNA sample is diluted at least 10 times in the final PCR tube, to avoid inhibition of the PCR by the RT buffer. The example given here is a cDNA dilution of 1:4 followed by a dilution of 2:5 in the PCR, resulting in a total dilution of 1:10 (v/v).

28. Vortex the 2x SsoAdvanced SYBR Green Supermix and spin down.
29. Vortex the forward and reverse primer well for at least 10 seconds and spin down.
30. Prepare a mastermix for each assay as described below.
    In this example, the mastermix is calculated for 10 samples (2 buffer treated control samples, 2 RNase R treated samples, 1 NTC, with qPCR duplicates), with a 12-sample excess. The excess is to allow the mastermix to be distributed using a repetition pipet. If you are using a regular pipet, a 10% excess should be sufficient for the mastermix. Always try to avoid pipetting small volumes (<2 µl).

	volume (µl) for 1 sample	**volume (µl) for mastermix for 10 samples (+ 12 excess)**
2x SsoAdvanced SYBR Green I Supermix	2.50	55.0
forward primer (5 µM)	0.25	5.5
reverse primer (5 µM)	0.25	5.5
total	3.00	66.0

31. Vortex each mastermix well for at least 10 seconds and spin down.
32. Distribute 3 µl of the mastermix for each assay in the correct well of the 384-well plate (using a repetition pipet). Distribute 2 µl of the samples (treated, control, water) in the correct wells using a multichannel pipet.

33. Seal the plate well and centrifuge the plate for 5 minutes at 400 g, room temperature.
34. Program the qPCR instrument as described below (here done for a LightCycler 480 II) and run the qPCR.

step		**temperature**	**time**	**ramp rate**	**acquisition mode**
pre-incubation		95 °C	2 min	4.8 °C/s	none
amplification	44 cycles	95 °C	5 sec	4.8 °C/s	none
		60 °C	30 sec	2.5 °C/s	none
		72 °C	1 sec	4.8 °C/s	single
melting curve		95 °C	5 sec	4.8 °C/s	none
		60 °C	1 min	2.5 °C/s	none
		95 °C		0.11 °C/s	continuous (5 per °C)
cooling		37 °C	3 min	2.5 °C/s	none

*See **Understanding Results** and Figure 3 on how to interpret the data. As mentioned earlier, RNase R treatment will not completely degrade all linear RNAs, but RT-qPCR will reveal which transcripts are RNase R susceptible (and, therefore, linear) and which ones are RNase R resistant (and, therefore, circular).*

Note that the melting peak should be the same in all samples but may differ from the melting peak of the synthetic DNA template positive controls, as these are generally shorter than the circRNA-based amplicons.

[*Figure 3 near here]

COMMENTARY

BACKGROUND INFORMATION

Recently, circRNAs have come into the spotlight due to their potential regulatory function and their association with various diseases (Meng et al., 2017). Large-scale detection of circRNAs is typically based on RNA sequencing data, but an orthogonal validation of specific circRNAs of interest is required for further research (Szabo and Salzman, 2016). For this, RT-qPCR with primers targeting the BSJ is most often performed. However, linear transcripts can lead to false-positive signals both in RNA sequencing as well as in RT-qPCR

experiments due to endogenous trans-splicing (Pfafenrot and Preußer, 2019). Therefore, it is essential to perform a validation step after using RNase R, an exonuclease that selectively degrades linear RNAs (Vincent and Deutscher, 2006; Jeck et al., 2013; Memczak et al., 2013). Here, we describe an RNase R treatment protocol that is used in combination with RT-qPCR to validate circRNAs. Of note, the same RNase R treatment protocol can also be used for treatment of RNA for RNA sequencing purposes, as we have successfully performed in combination with the NEBNext Ultra II RNA library preparation (#E7775, New England BioLabs) (data not shown).

Protocol optimization

A first step in the optimization of the protocol described here was the evaluation of the buffer cleanup step after RNase R treatment. This mandatory cleanup step is generally not described in RNase R articles (for example (Jeck and Sharpless, 2014; Memczak et al., 2013)), but is essential for the good performance of the subsequent RT step. Of note, compared to the miRNeasy Mini Kit (217004, QIAGEN) cleanup method (Panda and Gorospe, 2018) or using the RNA cleanup and concentrator (RCC-100, Zymo) (Sekar et al., 2019), our method of ultrafiltration using the Vivacon 500 Hydrosart 10 kDa columns (VN01H02, Sartorius Lab) is quicker, cheaper, and does not result in loss of RNA.

The purpose of the RNase R treatment is not to completely degrade linear RNA but to create the maximal reduction possible of linear RNAs while maintaining the levels of circRNAs intact. After testing increasing RNase R enzyme concentrations (1 U, 3 U, and 10 U per µg input RNA) and increasing incubation times (15 min, 30 min, 45 min, and 60 min) to treat 1 µg RNA, we concluded that a 15 min incubation time with an RNase R concentration of 1 U is optimal (among the conditions tested) to preserve circRNAs and degrade linear RNAs as much as possible from human RNA samples. Under these conditions, 99.7% of linear RNA was degraded. Furthermore, a wide range of RNA input amounts (10 ng, 100 ng, 1000 ng) have been tested with successful results, providing flexibility for the user depending on the available RNA amount or the need of RNase R treated RNA. These optimizations resulted in the protocol described here.

Limitations

Although we encourage using RNase R treatment of RNA samples for circRNA validation, it is important to keep in mind the limitations of the technique. For instance, while false-positive results can be caused by endogenous trans-splicing (which is verified with an RNase R treatment) (Pfafenrot and Preußer, 2019), they can also result from template-switching events of RT enzymes. As the RT step is performed after RNase R treatment, those false-positive results cannot be verified with RNase R. A possible solution is to validate circRNAs using two different RT enzymes, assuming both enzymes will not produce the same template-switching artefact (Szabo and Salzman, 2016). Furthermore, some known *bona fide* circRNAs seem to be degraded by RNase R due to their bigger size. For this, alternative methods for circRNA detection can be used, such as northern blot (Schneider et al., 2018).

CRITICAL PARAMETERS

For the circRNA BSJ sequence that is used for circRNA primer design, it is advised to double check the exact start and end nucleotides and ensure there is no additional or missing nucleotides due to conversion of 0-based/1-based annotation systems. CircRNA pipelines often report their results in the 0-based BED file format, whereas the UCSC genome browser (Haeussler eal., 2019) and Ensembl (Yates et al., 2019) use a 1-based format.

The RNA used for this protocol should be intact total RNA. The quality of the RNA can be assessed by inspecting the 18S and 28S peaks on agarose gel or capillary electrophoresis. As a rough guideline, an RNA integrity number (RIN) of at least 7 is recommended. Furthermore, if the RNA isolation is not performed correctly, the presence of left-over endonucleases could degrade the circRNAs (and linear RNAs) during the RNase R incubation step.

We have validated the protocol for RNA input amounts of 10 up to 1000 ng. Outside that range, the user should validate the efficacy of RNase R in terms of preservation of circRNA and degradation of linear RNA.

As previously emphasized, it is essential to perform a buffer cleanup step after RNase R treatment, to prevent RT inhibition by the RNase R buffer. When the cleanup step is omitted, the Cq values of linear control genes increase in the buffer control-treated

sample compared to the untreated sample (data not shown), which can incorrectly be interpreted as degradation of linear control genes (and circRNA).

TROUBLESHOOTING

No optimal assay design for circRNA

If no optimal assay can be designed for a circRNA using the settings described in Basic Protocol 1, the criteria should be relaxed, starting with Tm, delta Tm, and SNP presence. The Tm and delta Tm can be relaxed to 57-61 °C and 3 °C, respectively. SNPs can be allowed in the first half of the primer (5' end) (Lefever et al., 2013), and the amplicon size can be extended to 300. If still no primers are found, the template sequence can be broadened to 400 nucleotides (in total, e.g., 350 and 50 on both sides of the BSJ) wth an amplicon size of <350 nucleotides.

Suboptimal PCR efficiency

When testing new primers on a dilution series, the primer efficiency should be between 90 % and 110%. If efficiency in that range is not observed, the pipette should be calibrated, the highest dilution point(s) could be removed (for low abundant targets), or the assay should be redesigned (a suboptimal PCR efficiency is often caused by amplicon secondary structure).

Replicate variability

In general, we aim for less than a 0.5 Cq value difference when using qPCR replicates. When using RT or RNase R replicates, higher variability is expected. If the replicates do not meet these criteria, pipetting skills may need to be improved or more input material can be used (because of stochastic variation, replicate variability increases at lower template concentration).

More than one specific melting peak is observed

When more than one melting peak is observed during qPCR (using a DNA-binding dye based detection such as SYBR green I), first verify that the amplicon does not melt in two different domains using uMelt (Dwight et al., 2011). If it does, the assay should be redesigned. This can also be confirmed based on electrophoresis of the amplicon. If two

or more amplicons or deviation of amplicon length are observed, the assay should be redesigned.

Degradation of bona fide circRNA

If *bona fide* circRNAs previously described in the literature with alternative methods are degraded and cannot be observed after RNase R treatment, use a lower concentration of RNase R or reduce the RNase R incubation time.

UNDERSTANDING RESULTS

Using the optimized RNase R treatment protocol described here, linear controls should be degraded as expected (Figure 3A), and a Cq increase should be observed when comparing control and RNase R-treated samples.

For *bona fide* circRNAs, no significant difference in Cq should be observed between the treated and untreated samples. The example data shown in Figure 3B, shows this for hsa_circ_0000118, hsa_circ_0000119, hsa_circ_0000284, and hsa_circ_0000567

In the case of hsa_circ_0111784, data derived from the approach described in this article suggest that it is a false positive circRNA. This circRNA was identified in our SW480 RNA sequencing data by the circRNA detection tool find_circ (Memczak et al., 2013), with 1123 BSJ counts. Furthermore, it was previously detected in different human neural tissues (also by find_circ) (Rybak-Wolf et al., 2014) and is present in the circRNA databases circBank (Liu et al., 2019), circbase (Glažar et al., 2014), and circAtlas (Wu et al., 2020). Surprisingly, upon RNase R treatment of SW480 RNA followed by RT-qPCR, it behaved like a linear RNA (Figure 3C), exhibiting an increase in the Cq between the treated and untreated samples, suggesting that this circRNA is a false-positive result, and underscoring that RNase R treatment is an essential validation step in circRNA studies.

The use of circRNAs as reference targets for RT-qPCR data normalization

To validate circRNAs using RNase R and RT-qPCR, raw Cq values can be used (Fig 3). However, for quantification and comparison of circRNAs in different biological samples,

a normalization step is needed. For regular gene expression studies using RT-qPCR, multiple validated reference genes are required to account for experimentally induced variation, according to the MIQE guidelines (Bustin et al., 2009) and ISO 20395:2019. This internal control should not vary in the cells under investigation or in response to a treatment of interest (Vandesompele et al., 2002). When using RNase R, linear reference genes cannot be used, as they are degraded. Alternatively, stably expressed circRNAs should be selected. For this, the geNorm algorithm in qbase+ (Biogazelle, Zwijnaarde, Belgium; www.qbaseplus.com) can be used to calculate the average expression stability values (M-values) of the reference targets and the optimal number of reference targets for that experiment (based on the V-value). Reference targets are considered stable when their M-value is below 0.5. The optimal number of reference targets is achieved when the V-value drops below 0.15 (minimum two, sometimes more, depending on the variability of the samples).

For example, for SW480 experiments (total cell line RNA, with RNase R treatment according to the optimized protocol described here, including both treatment and qPCR duplicates), the M-values for hsa_circ_0000118, hsa_circ_0000119, hsa_circ_0000284, and hsa_circ_0000567 were all below 0.5 (Figure 4). Furthermore, geNorm identified the optimal number of reference targets for this experiment to be two: hsa_circ_0000119 and hsa_circ_0000284. For similar experiments using HLF and Human Universal Reference RNA, the same two circRNAs were reported as suitable stable reference targets. For experiments in NCI-H23, three stable reference targets were proposed: hsa_circ_0000118, hsa_circ_0000119, and hsa_circ_0000567. Of note, reference target stability depends on multiple factors, such as the sample type and treatment and should, therefore, always be verified for the particular experimental set-up.

[*Figure 4 near here]

TIME CONSIDERATIONS

Basic Protocol 1 (circRNA primer design and primer validation) is dependent on the delivery of the circRNA primers and synthetic DNA template positive controls. CircRNA primer design can be completed in half a day, depending on the number of circRNAs to

validate. Once the primers and synthetic controls have arrived, the dilution of the synthetic controls and efficiency qPCR can be performed in half a day.

Basic Protocol 2 (RNase R treatment, buffer cleanup, and RT-qPCR) can easily be completed in one day. Assembly of the reaction mixtures for RNase R treatment takes 5-10 minutes. This is followed by an incubation period of 15 minutes, during which the ultrafiltration columns can be pretreated. The following steps of the ultrafiltration of the samples should take up no more than 30 minutes to one hour, depending on the number of samples. Next, the RT step takes approximately 30 minutes (assembly of reaction mixture and incubation). Preparing the mastermixes for qPCR, and distributing the mixes and samples in the plate can take 1-2 hours, depending on the number of samples and the number of assays. Lastly, running the qPCR takes approximately 1-1.5 hour.

CONFLICT OF INTEREST STATEMENT:

J.V. is co-founder and CSO at Biogazelle, which provides the qbase+ software.

DATA AVAILABILITY STATEMENT:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS

This work was supported by a Foundation Against Cancer grant (STK F/2018/1267), Standup Against Cancer grant (STIVLK2018000601) and Concerted Research Action of Ghent University (BOF16/GOA/023) and the Research Foundation - Flanders (FWO 1253321N).

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INTERNET RESOURCES

http://www.unafold.org/mfold/applications/dna-folding-form.php

UNAfold offers a tool for nucleic acid folding and hybridization prediction (mfold), which can be used to verify the absence of secondary structures of the amplicon.

http://bisearch.enzim.hu/?m=genompsearch

Bisearch offers an ePCR tool to verify the target specificity of your primers.

https://www.dna-utah.org/umelt/quartz/um.php

UMelt is a tool that predicts the melting curve of a specific amplicon.

FIGURES

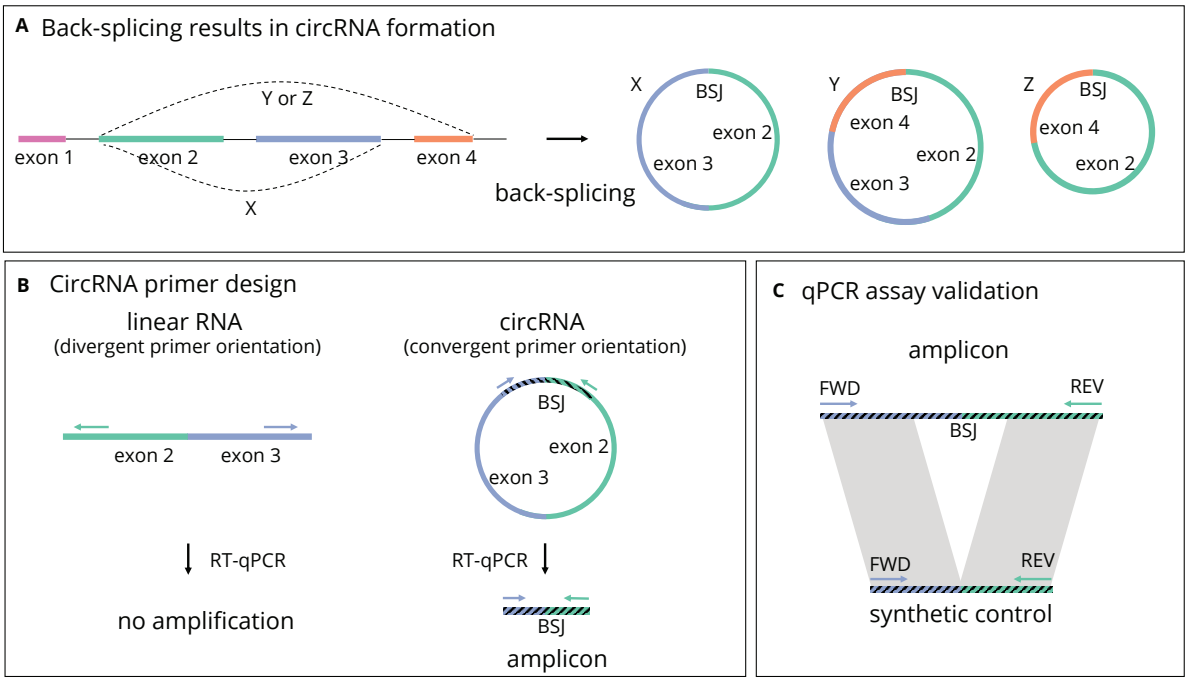

Figure 1. **CircRNAs backsplicing, primer design, and primer validation**. **A** One gene can give rise to multiple circRNAs (X, Y, Z) varying in BSJ sequence, and in exon and intron composition. **B** BSJ-spanning primer design for circRNA detection. CircRNA primers are designed by selecting the 300-nucleotide region surrounding the putative BSJ. The primers should be divergent when annealing to linear RNA (facing away from each other) and convergent when annealing to circRNA (facing towards each other), to ensure specific circRNA detection. **C** Design of synthetic DNA template positive controls for circRNA primers. The first and last 30 nucleotides of the amplicon can be concatenated to generate a positive control template. This control does not have to include the circRNA BSJ; even without it, it is still perfectly adequate to test the amplification efficiency of the primers. FWD: forward primer, REV: reverse primer.

A

		point 1		point 2		point 3		point 4		point 5		point 6		carrier		NTC		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	...
circ 1	A	pt_1	pt_1	pt_2	pt_2	pt_3	pt_3	pt_4	pt_4	pt_5	pt_5	pt_6	pt_6	carrier	carrier	NTC	NTC	
circ 2	B	pt_1	pt_1	pt_2	pt_2	pt_3	pt_3	pt_4	pt_4	pt_5	pt_5	pt_6	pt_6	carrier	carrier	NTC	NTC	
circ 3	C	pt_1	pt_1	pt_2	pt_2	pt_3	pt_3	pt_4	pt_4	pt_5	pt_5	pt_6	pt_6	carrier	carrier	NTC	NTC	
circ 4	D	pt_1	pt_1	pt_2	pt_2	pt_3	pt_3	pt_4	pt_4	pt_5	pt_5	pt_6	pt_6	carrier	carrier	NTC	NTC	
	...																	

B

		buffer treated control				RNase R treated				NTC		
		1	2	3	4	5	6	7	8	9	10	...
circ 1	A	B_1	B_1	B_2	B_2	T_1	T_1	T_2	T_2	NTC	NTC	
circ 2	B	B_1	B_1	B_2	B_2	T_1	T_1	T_2	T_2	NTC	NTC	
circ 3	C	B_1	B_1	B_2	B_2	T_1	T_1	T_2	T_2	NTC	NTC	
circ 4	D	B_1	B_1	B_2	B_2	T_1	T_1	T_2	T_2	NTC	NTC	
lin 1	F	B_1	B_1	B_2	B_2	T_1	T_1	T_2	T_2	NTC	NTC	
lin 2	G	B_1	B_1	B_2	B_2	T_1	T_1	T_2	T_2	NTC	NTC	
	...											

Figure 2. **Examples of qPCR run lay-outs. A** Run lay-out for primer validation. CircRNA primers can be validated using a 6-point, 10-fold dilution series of synthetic DNA template positive controls (point 1-6). Furthermore, a carrier RNA (tRNA from Brewer's Yeast) negative control and no-template control (NTC) is included. **B** Run lay-out for circRNA validation. Buffer treated control samples (B) and RNase R treated samples (T), *in duplo* (treatment replicates, _1 and _2). Each circRNA and linear assay is measured twice (qPCR replicates) in the four samples shown here as an example**.** Furthermore, an NTC is included.

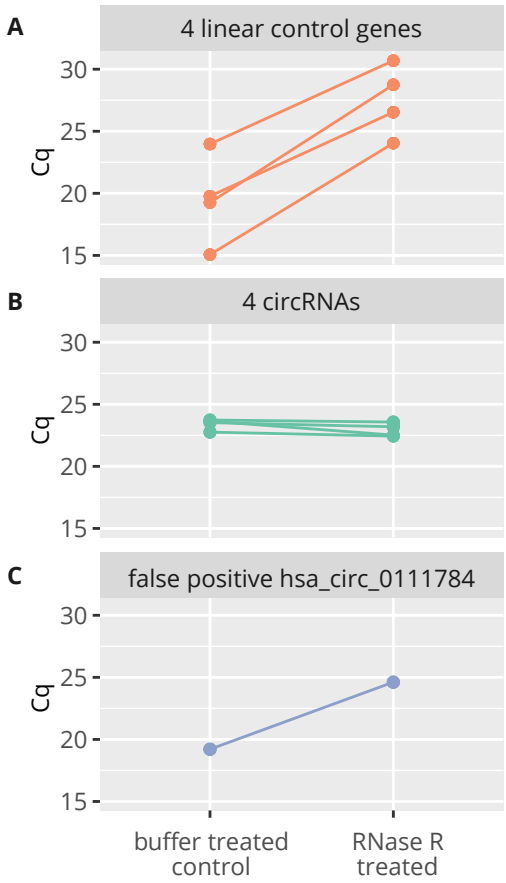

Figure 3. **RNase R treatment is an essential step in circRNA validation**. For this experiment, total RNA was isolated from SW480 cell line, and the RNase R protocol was performed as described above. All treatments were performed *in duplo*, and the average Cq of both treatment replicates calculated and presented here. **A** All linear control genes (*ACTB*, *B2M*, *HPRT1* and *TBP*) are degraded upon RNase R treatment. **B** *Bona fide* circRNAs (hsa_circ_0000118, hsa_circ_0000119, hsa_circ_0000284, hsa_circ_0000567) are relatively stable upon RNase R treatment. In this sample data, we observed a mean Cq increase of 8.17 (sd = 1.42) for the linear targets, and a mean increase in Cq of 0.49 (sd = 0.43) for the circRNAs. **C** Hsa_circ_0111784 is degraded upon RNase R treatment, suggesting this is a false-positive result. In this example, we observed a Cq increase of 5.3 for this circRNA. Note, however, that this circRNA has been described in multiple databases and was also found in our dataset by find_circ.

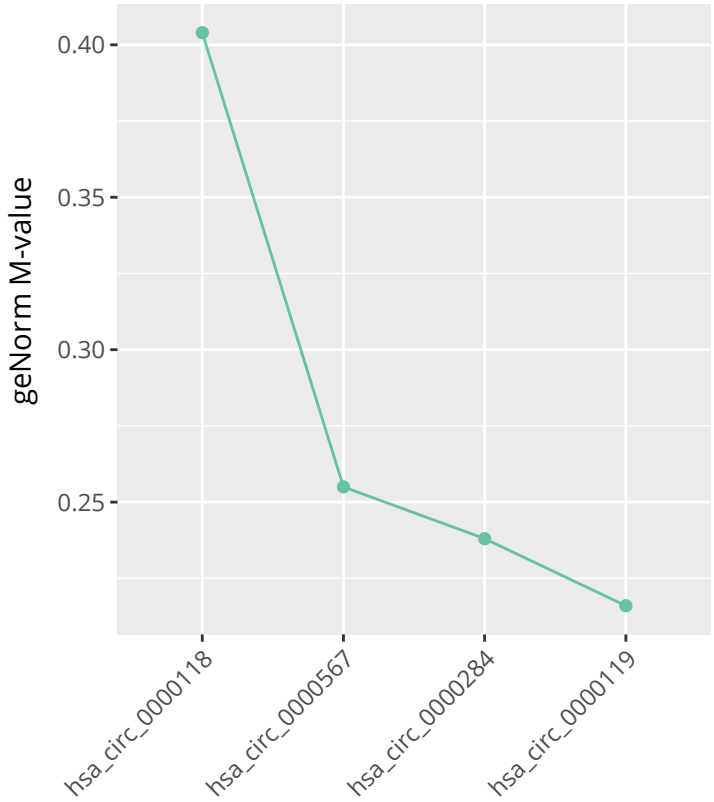

Figure 4. **circRNAs as reference targets for RT-qPCR data normalization.** Here, SW480 cells were grown according to ATCC guidelines, and the stability of different circRNAs as reference genes was evaluated in buffer-treated and RNase R-treated RNA samples. Using the geNorm algorithm, the reference target stability (M-value, shown here) and optimal number of reference targets was calculated (V-value) for four selected circRNAs. Based on the geNorm M-value and V-value (not shown), hsa_circ_0000119 and hsa_circ_0000284 were selected as stable reference targets for the SW480 experiments.

Tables

circRNA id (hg38, 0-based[a])	circRNA name (circBase[b])	host gene
chr1:117402185-117420649	hsa_circ_0000118	MAN1A2
chr1:117402185-117442325	hsa_circ_0000119	MAN1A2
chr11:33286412-33287511	hsa_circ_0000284	HIPK3
chr14:99458278-99465813	hsa_circ_0000567	SETD3
chr1:207652546-207752309	hsa_circ_0111784	CD46

Table 1 CircRNAs selected for protocol optimization experiments and sample data. Primer sequences are available in Supplementary Table 1.

[a] Genomic positions are reported in the 0-based format as described in the BED file format.

[b] There is currently no uniform circRNA annotation system (Vromman et al., 2020). As different names are sometimes used for the same circRNA, the BSJ position should always

be mentioned, as this is the only unique identifier of a circRNA. Here, the circBase annotation is used (Glažar et al., 2014).

Supplementary tables

circRNA BSJ position	**forward primer**	**reverse primer**
chr1:117402185-117420649	AGGTGTCTGTGTTTGAAGTC	TGCTCGAATTTCCTCTCTTG
chr1:117402185-117442325	CTTGGGCAATGGTGAATTTG	TGCTCGAATTTCCTCTCTTG
chr1:207652546-207752309	TTGCTGCTGTACTCCTTCT	AATAGTATGGGTGGCAAGAG
chr11:33286412-33287511	ATTATGTTGGTGGATCCTGTT	ATATGGTGGGTAGACCAAGA
chr14:99458278-99465813	CTCGACTCTTCTTACCCATTT	AGTGAATATGACACTCCTCTCT
linear target	**forward primer**	**reverse primer**
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC

Supplementary Table 1 Primer sequences of circRNAs and linear control genes

BSJ position (hg38)	synthetic control (green and blue: first and last 30 nucleotides of amplicon, respectively)
chr1:117402185-117420649	AGGTGTCTGTGTTTGAAGTCAACATTCGATTTAAGAAAGTCAAGAGAGGAAATTCGAGCA
chr1:117402185-117442325	CTTGGGCAATGGTGAATTTGAAAAGGGGAATTAAGAAAGTCAAGAGAGGAAATTCGAGCA
chr1:207652546-207752309	TTGCTGCTGTACTCCTTCTCCGGATGCCTATACGTACCTCCTCTTGCCACCCATACTATT
chr11:33286412-33287511	ATTATGTTGGTGGATCCTGTTCGGCAGCCTGCCTCACAAGTCTTGGTCTACCCACCATAT
chr14:99458278-99465813	CTCGACTCTTCTTACCCATTTTTCTGACCTTTCAAAGTAGAGAGGAGTGTCATATTCACT

Supplementary Table 2 Sequences of synthetic DNA template positive controls of selected circRNAs