Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59760R2
Full Title:	Use of Alu-element containing minigenes to analyze circular RNAs
Keywords:	circular RNA alternative splicing reporter gene minigene mRNA transfection assay RT-PCR
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lexington, KY

TITLE:

Use of *Alu*-Element Containing Minigenes to Analyze Circular RNAs

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KEYWORDS:
circular RNAs, alternative splicing, reporter gene, minigene, RNA, mRNA

SUMMARY:
We clone and analyze reporter genes generating circular RNAs. These reporter genes are larger than constructs to analyze linear splicing and contain *Alu* elements. To investigate the circular RNAs, the constructs are transfected into cells and resulting RNA is analyzed using RT-PCR after removal of linear RNA.

ABSTRACT:
In addition to linear mRNAs, many eukaryotic genes generate circular RNAs. Most circular RNAs are generated by joining a 5' splice site with an upstream 3' splice site within a pre-mRNA, a process called back-splicing. This circularization is likely aided by secondary structures in the pre-mRNA that bring the splice sites into close proximity. In human genes, *Alu* elements are thought to promote these secondary RNA structures, as *Alu* elements are abundant and exhibit base complementarities with each other when present in opposite directions in pre-mRNA. Here, we describe the generation and analysis of large, *Alu* element containing reporter genes that form circular RNAs. Through optimization of cloning protocols, reporter genes with up to 20 kb insert length can be generated. Their analysis in co-transfection experiments allows the identification of regulatory factors. Thus, this method can identify RNA sequences and cellular components involved in circular RNA formation.

INTRODUCTION:

Circular RNAs

Circular RNAs (circRNAs) are covalently closed single stranded RNAs that are expressed in most organisms. They are generated by joining a downstream 5' splice site to an upstream 3' splice site, and is called back-splicing (**Figure 1A**)[1]. Sequences in the pre-mRNA that exhibit base complementary as short as 30-40 nt bring back-splice sites into proper alignment for circRNA formation[2]. In humans, *Alu* elements[1], representing about 11% of the genome[3], form extensive double stranded RNA structures in pre-mRNA due to their self-complementarity[4,5] and thus promote the formation of circRNAs[1].

Currently, three major functions of circRNAs have been described. Some circRNAs bind microRNAs (miRNAs) and through sequestration act like miRNA sponges[6]. CircRNAs have been implicated in transcriptional and post transcriptional regulation, through competition with linear splicing[7] or modulation of transcription factor activity[8]. Finally, circRNAs contain short open reading frames and proof of principle studies show that they can be translated[9,10]. However, the function of most circRNAs remains enigmatic. The majority of circular RNAs have been detected using next-generation sequencing methods[11]. Detailed analyses of individual genes using targeted RT-PCR approaches reveal that a large number of circular RNAs remains to be discovered[12].

Use of reporter genes to analyze pre-mRNA processing

The analysis of mRNA derived from DNA reporter constructs transfected into cells is a well-established method to study alternative pre-mRNA splicing, which can be applied to circular RNAs. In general, the alternative exon and its surrounding introns and constitutive exons are amplified and cloned into a eukaryotic expression vector. Frequently, the introns are shortened. The constructs are transfected into eukaryotic cells and usually analyzed by RT-PCR[13,14]. This approach has been extensively used to map regulatory splicing sites and trans-acting factors in co-transfection experiments[13,15-18]. In addition, the generation of protein-expressing minigenes allowed for screening of substances that change alternative splicing[19,20].

The method has been applied to circular RNAs. Currently, at least 12 minigene backbones have been described in the literature and are summarized in **Table 1**. With the exception of the tRNA based expression system[21,22], they are all dependent on polymerase II promoters. Here, we describe a method to generate human reporter minigenes to determine cis and trans-acting factors involved in the generation of circular RNAs. An overview of the method using sequences of a published reporter gene[23] is shown in **Figure 1**.

PROTOCOL:

1. Design of the constructs

1.1. Use the UCSC genome browser[24] to identify repetitive elements necessary for circular RNA formation and incorporate them in the constructs. Importantly, primers for amplification need to be outside the repetitive elements.

1.2. Paste the circular RNA sequence (**Supplemental Figure 1** is a test sequence) into https://genome.ucsc.edu/cgi-bin/hgBlat?command=start and select the right organism. Submit the sequence and go to browser view, zoom out 1.5x or as appropriate (**Figure 2A**).

NOTE: The search sequence appears in the top line (**Figure 2A, 1**). Depending on the order of the exons in the circular RNA, BLAT will not connect all the exons. In this example, exon 12 (**Figure 2A, 4**) is not connected to 11 (**Figure 2A, 2, 3**), because exon 12 is upstream of exon 11 in the circular RNA sequence (**Supplemental Figure**

1). The repetitive elements are in the 'repeat masker' track, indicated by boxes, where black to gray color indicates the evolutionary conservation (**Figure 2A, 5**).

1.3. Mouse over the repetitive elements to identify their subtype in a floating window. *Alu* elements are in the SINE (short interspersed nuclear element) line. Use the 'default tracks' button under the window to reset the browser if a different picture than **Figure 2** is obtained.

NOTE: Mousing over the exons in the gene display generates a window with exon numbers that are computer generated. These numbers do not correspond to the exon numbering established in the literature and also change between isoforms.

2. Select the sequence to be cloned in an expression vector

2.1. Download the DNA sequence shown in the window by going to **View** → **DNA** on the top line of the UCSC genome browser. In the **Sequencing Formatting Option**, select **Extended Case/Color Options**.

2.2. Select the default case as **Lower** and select toggle case for **NCBI refseq**. Select underline, and bold, and italic for **Repeat Masker**. Click **Submit**. There will be exons as capital letters and introns as small letters. Check the exon/intron borders.

2.3. In this example there is a 'ccctttacCTTTTT' sequence, indicating that the browser shows the reverse complement. If this is the case, go back and select the reverse complement box until seeing the correct exon intron borders (agEXONgt), in this example AAAAAGgtaaaggg.

2.4. Copy the file with the correct orientation (internal exons are surrounded by intronic ag…gt) into a word processing document and highlight the exons (**Supplemental Figure 2**).

NOTE: In this example, the genomic fragment encompassing exons 9-12 is around 24 kb and thus too large to be amplified from genomic DNA. Therefore, each exon surrounded by about 1 kb intronic region is individually amplified and these four fragments assembled in a cloning vector.

2.5. Select fragments to be amplified (exon ± 500 nt intron). Make sure that the intron does not begin or end in a repetitive region, as primers in these regions will not amplify specific sequences. The selected regions are shown in **Figure 2B**, and their sequences are shown in **Supplemental Figure 3**.

NOTE: In general, the larger the constructs, the more difficult the cloning will be. The fragments can be assembled either step wise (i.e., exon 9 is combined with the vector and in the next step exon 10 is introduced into this construct via cloning until all exons are in place), or alternatively, all fragments are assembled simultaneously. A stepwise approach always works but requires more time. A simultaneous assembly does not always work and depends how well the individual fragments can be amplified from

genomic DNA and on the overall size of the construct. We therefore usually start with both approaches simultaneously.

3. Design primers for cloning

3.1. Use a web tool (https://nebuilder.neb.com/#!/) to design the primers for cloning. For example, enter fragments 9, 10, 11 and 12 and the vector sequence (**Supplemental Figure 3**) to this tool.

3.2. For the vector sequence, add the insertion site as the last nucleotide and subsequently add the fragments. Since the vector numbering does not start with a given insertion site, the site of insertion in the vector is located and the downstream part is put in in front of the upstream sequence. In this example the inserts start directly after the HindIII [AAGCTT] site and ends directly after the PmeI [GTTTAAAC] site of pcDNA3.1. The sequence from cccgctgatcag … ccgtaaaaaggccgc is pasted before position 1 in the pcDNA3.1 sequence (gttgctggcgtttttcc …).

3.3. Adjust primers if their melting points are more than 4 ºC apart and do not work in amplification. The assembly of the fragments and primer sequences designed are shown in **Supplemental Figure 4.** Primers for cloning can also be designed manually[25].

4. PCR and amplicon detection

4.1. Standard PCR Reaction: Make a reaction mix for total volume of 50 µL per reaction. Below is the recipe for one reaction using polymerase 1:

10 µL of 5x Reaction Buffer
1 µL of 10 mM dNTPs
2.5 µL of 10 µM Forward Primer
2.5 µL of 10 µM Reverse Primer
0.5 µL of Polymerase 1
32.5 µL of Nuclease free H_2O

4.1.1. Optionally, add 10 µL of 5x GC Enhancer if the product has high GC content; make a separate mix containing GC Enhancer to test PCR reaction.

4.2. Aliquot 49 µL of the mix into PCR tubes per reaction sample.

4.3. Add 1 µL of DNA to the PCR, the amount ranges from 10 pg to 1 ng.

4.4. Spin down the samples to remove residue off the sides and place them in a PCR machine. Use the same machine as well as same spots in the machine when optimizing the PCR conditions.

5. Optimization for longer DNA fragments for use of different polymerases

5.1. Temperature

5.1.1. Optimize the long-range Polymerase 2.

5.1.1.1. Use a lower denaturation temperature (Polymerase 1: 98 ºC, Polymerase 2: 94 ºC).

5.1.1.2. Use a longer denaturation time (Polymerase 1: 10 s, Polymerase 2: 30 s).

5.1.1.3. Use a longer annealing time (Polymerase 1: 30 s, Polymerase 2: 60 s).

5.1.1.4. Use a longer extension time (Polymerase 1: 30 s/kb, Polymerase 2: 50 s/kb).

5.1.1.5. Use a lower extension temperature (Polymerase 1: 72 ºC, Polymerase 2: 65 ºC).

5.1.1.6. Use an annealing temperature 5 ºC below the Tm of the primers.

5.1.2. Optimize the primer concentrations (5x less to 5x more than the original primer concentration). Optimize DNA concentrations from 10 pg to 50 pg, 100 pg, 1 ng, 5 ng, 10 ng.

5.1.3. As an example of a PCR program to amplify a 15 kb product size using Polymerase 2, perform an initial denaturation at 94 ºC for 30 s, DNA denaturation at 94 ºC for 30 s followed by annealing at 58 ºC for 30 s, and DNA extension at 65 ºC for 12 min 30 s. Perform a final extension at 65 ºC for 10 min with a final 4 ºC hold.

NOTE: The annealing temperature (Ta) specific for the primers determined by web-based temperature calculations that are specific for each polymerase. Extension times may vary and need to be optimized if fragments are larger than 10 kb.

5.2. Extension times and DNA concentrations

5.2.1. Use longer extension times for DNA fragments over 6 kb: 1 min per 1 kb. Longer fragments should use less DNA.

5.2.2. Perform dilutions of DNA to find the optimum DNA concentration for amplification, usually 1 pg to 1 ng for plasmids or 1 ng to 1 µg for genomic DNA.

NOTE: For most cloning use polymerase 1, engineered by fusing the Sso7d DNA binding domain to a proprietary thermostable DNA polymerase[26]. The polymerase has a low error rate and due to the Sso7d domain a high processivity, needed to amplify large (10-15 kb) genomic fragments. Due to this large fragment size, enzymatic assembly of DNA molecules[27] is used for insertion into vectors, as this method does not require restriction enzymes. The amplification of fragments longer than 15 kb gets increasingly difficult with the Q5 polymerase. For large fragment amplification use polymerase 2 made from *pyrococcus*-like proofreading polymerase fused to Sso7d or the long range PCR kit that gives, however, higher error rates.

6. Purification of PCR products for cloning

6.1. Run half of the PCR products on 1% agarose gels containing an intercalating nucleic acid stain (e.g., 1x GelGreen). Visualize the stained gels on a dark reader transilluminator. This stained DNA does not run true to size.

NOTE: GelGreen intercalates into DNA, similar to ethidium bromide, but it is excited by light around 500 nm (cyan), a wavelength that does not damage DNA, which highly improves cloning.

6.2. In parallel, run half of the PCR products on a 1% gel that is stained post-run with ethidium bromide (**Figure 3A,B**).

6.3. Excise bands of the right size from the stained gel (step 6.1) and isolate DNA using a gel and PCR cleanup kit. In deviation from its standard protocol, elute the DNA in only 20 µL of double distilled water, as usually the DNA concentrations are low.

6.4. Prior to cloning, check the isolated DNA on an agarose gel for concentration and integrity (**Figure 3B**). Aim for a 2:1 molar insert to vector ratio. When using several inserts, the ratio is 2:2:2:1.

7. DNA assembly and clone detection

NOTE: Cloning is done using an enzymatic DNA assembly kit, with minor modifications. The assembly is performed for 60 min at 50 ºC and generally the lower range of DNA is used for assembly (20-100 fmol/20 µL reaction). The whole reaction mix is added to chemical competent cells and the whole cells plated out on a 6 cm agar plate.

7.1. Combine the vector and insert in a 1:2 molar ratio (20-500 fmol each) in 10 µL of water.

7.2. Add 10 µL of DNA Assembly Master Mix.

7.3. Incubate samples for 60 minutes at 50 ºC.

7.4. Next, transform competent cells with the total assembly reaction. Here, use *E. coli* strain 1 cells for shorter constructs or *E. coli* strain 2 cells for longer or unstable constructs. Cells should be in a 50 µL volume.

7.5. Thaw cells on ice and add 2 µL of the chilled assembled product to the competent cells. Mix by gently flicking the tube 4-5 times. Do not vortex.

7.6. Let the mixture sit on ice for 30 min.

7.7. Heat shock at 42 ºC for 30 s. Do not mix.

7.8. Transfer the tube back to ice for 2 min.

7.9. Add 950 µL of room-temperature SOC Media to the tube.

7.10. Incubate the reaction tube at 37 ºC for 60 min. Shake vigorously (300 rpm).

7.11. Warm selection plates with the appropriate antibiotic during incubation at 37 ºC.

7.12. Pellet the cells through centrifugation (10,000 x *g*, 30 s) and plate out ¼ and ¾ of cells on two selection plates and incubate at 37 ºC overnight.

8. Validation of clones

8.1. Use colony PCR, employing PCR primers spanning the assembly sites (**Figure 1C**) for detection.

8.2. Take a sterile toothpick and touch a single bacterial colony.

8.3. Touch the bottom of a PCR tube with the toothpick that has the colony and then streak the toothpick on an antibiotic agar plate, incubate the streaked plate at 37 ºC or room temperature for sensitive constructs overnight.

8.4. Overlay the PCR tube touched with the colony with a PCR mix containing the detection primers. These are designed using primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/). The program has the option to force primer design across a defined sequence using the “[ccc]” signs to select primers across the assembly junction. DNA from positive strains is isolated and verified by sequencing.

9. Analysis of circular RNA expressing reporter genes

9.1. For analysis, transfect reporter genes into eukaryotic cells. Here, use HEK293 cells (ATTC #CRL-1573) as they give high transfection efficiency. To save costs, use PEI (polyethyleneimine) solutions.

9.2. For the PEI solution, dissolve linear polyethyleneimine hydrochloride (PEI) at 1 mg/mL in water at low pH, pH 2. Bring up the pH to 7 with NaOH. Sterile filter with 0.22 µm filters and store at 4 ºC.

9.3. Split cells into six wells (approximately 150,000 cells per well) and let them grow overnight in 10% FBS in DMEM media.

9.4. Aliquot 1 µg of the reporter gene in a sterile tube and add 200 µL of sterile filtered 150 mM NaCl. Mix with the DNA by vortexing.

9.5. Add PEI solution to this mix and vortex, briefly centrifuge to collect samples at bottom of tube. Use a ratio of 1 µg of DNA per 3 µL of PEI.

9.6. Incubate at room temperature for 10 min and then add directly to HEK 293 cells.

9.7. Incubate HEK293 cells at 37 ºC, 5% CO_2, overnight.

9.8. Isolate RNA for RT-PCR via an RNA isolation kit.

10. RNase R treatment to remove linear RNAs

10.1. Use 10 µg of total RNA in an RNase-free tube.

10.2. Add 10 µL of 10x RNase R buffer (0.2 M Tris-HCl (pH 8.0), 1 M KCl, 1 mM $MgCl_2$) to RNA.

10.3. Add RNase R to RNA.

10.4. Add 1 µL of glycol blue to the RNA and bring the volume up to 100 µL with sterile water.

10.5. Incubate samples at 37 ºC for 30 min.

10.6. Add 100 µL of phenol/chloroform and vortex for 1 min.

10.7. Centrifuge at 21,000 x *g* for 1 min to separate phases.

10.8. Take the supernatant (aqueous phase) and add 1 volume (around 80 µL of chloroform).

10.9. Vortex for 1 min, and then centrifuge for 1 min to separate phases.

10.10. Take supernatant, add 1:10 vol KAc and 2.5 vol ethanol, and precipitate at -20 ºC for 1-4 h. Centrifuge at 4 ºC for 30 min at full speed (21,000 x *g*). There will be a small blue pellet at the bottom.

10.11. Remove the supernatant, and wash with 80% ethanol. Let air dry for 5 min at room temperature, and dissolve in 10 µL water.

11. RT-PCR analysis

11.1. Use 1 µg of RNA per RT reaction.

11.2. Make reaction mix for a final total volume of 20 µL per reaction. For one reaction, use 1 µL of 10 mM dNTPs, 1 µL of 0.1 M Dithiothreitol (DTT), 4 µL of 5x First-Strand Buffer, and 0.5 µL of Reverse Transcriptase.

11.3. Aliquot 6.5 µL of reaction mix into new PCR tubes.

11.4. Mix up to 5 primers in one mix. However, some primers may mis-pair with other primers in the PCR (**Figure 5**). Primer sequences are in **Table 2**. We routinely use gene-specific exon-junction primers but priming with random hexamers is also possible.

11.5. Add 1 µL of 10 µM Reverse primer to desired RT reaction tube.

11.6. Add 1 µg of RNA to PCR reaction tube.

11.7. Add RNase-free H_2O up to a total volume of 20 µL.

11.8. Spin tubes down to remove residue on side of tubes and place in thermocycler.

11.9. Run the RT reaction in thermocycler at 50 ºC for 50 minutes.

11.10. Store RT cDNA at -20 °C or proceed to the PCR reaction.

REPRESENTATIVE RESULTS:

Reporter genes allow determination of regulatory factors that influence circular RNA formation. However, these reporter genes are large and contain repetitive elements that often make DNA constructs unstable. Due to their large size, it is often necessary to delete parts of the introns, which is achieved by amplifying genomic pieces containing the exons and smaller flanking intronic parts. These DNA pieces are enzymatically assembled, allowing construction without restriction enzymes.

The example of a circular RNA generated from the microtubule associated protein tau (MAPT) shows an application of the minigene approach to analyze circular RNAs. The tau 9→12 minigene used in this example was co-transfected with different splicing factors and the effect of these splicing factors was detected by RT-PCR (**Figure 6**). Different trans-acting factors influence both circular RNA and linear pre-mRNA formation. The experiment also shows that all the sequence elements necessary for circular RNA formation are localized in the cloned fragment.

Figure 1: Overview of the technique.

(**A**) A hypothetical gene is shown. Introns are lines, exons are boxes, *Alu* elements are smaller striped boxes. Backsplicing from exon C to A creates a circular RNA. The structure of this circular RNA is shown in panel (**E**). (**B**) To create a reporter gene, exons and surrounding introns (at least 500 nt on each side) are amplified. The constructs should contain repetitive elements, which are usually *Alu* elements in humans. An exon upstream of exon A was included to provide an additional *Alu* element. The genomic fragments will overlap with their flanking 25 nts. (**C**) Fragments are cloned into an expression vector, driven by a CMV promoter. The successful recombination is detected by detection primers and validated by sequencing. (**D**) Cells are transfected with this construct. (**E**) Circular RNA is isolated and (**F**) amplified using circular RNA specific primers, preferable exon junction primers. During PCR amplification, linear RNA can also be amplified (**G**). (**G**) Orientation of the primers used to detect circular RNAs. The forward primer is in sense orientation (i.e., has the same sequence as the RNA) and the reverse primer is in antisense orientation (i.e., is the reverse complement of the RNA). Note that different from RT-PCR for linear mRNAs, the reverse primer is upstream of the forward primer.

Figure 2: Selection of the sequence for minigene construction.
(**A**) Browser display after the sequence shown in **Supplemental Figure 1** is run against the human genomic database using BLAT. Literature exon numbers[36] are indicated in the gene display, they are different from the numbers given by the browser.
1. The aligned sequences are shown under 'YourSeq'
2-4. Note that due to the circularity of the RNA, BLAT does not connect all exons with lines as it does in linear RNA. Exons 10 and 11 (corresponding to 2 and 3) are connected, but exon 12 (corresponding to 4) is not connected to exon 11.
5. *Alu* elements are shown in the repetitive element track.
(**B**) Sequence alignment between the planned construct and genomic DNA.
6. The planned construct was run against the database using BLAT.
7. Note the inclusion of several *Alu* elements in the construct.

Figure 3: Example of the amplicons prior to cloning
(**A**) Optimized PCR products separated on a 1% agarose gel containing 1x GelGreen. The individual bands represent the PCR products that will be used in enzymatic DNA assembly. (**B**) The bands from (**A**) were cut out from the gel and purified. The purified PCR products were separated on a 1% agarose gel, which was subsequently stained with ethidium bromide.

Figure 4: Restriction analysis of reporter genes
The tau 9-12 minigene used as an example was cut with restriction enzymes indicated to rule out major recombinations. Lane 1: cut with NcoI expected sizes 735 bp, 3345 bp, 6266 bp, lane 2 cut with XbaI expected size 10346 bp, lane 3 cut with HindIII expected sizes 3951 bp, 6395 bp, lane 4 cut with SmaI expected sizes 1168 bp, 1688 bp, 2708 bp, 4782 bp.

Figure 5: Effect of primer multiplexing and RNase R treatment on circular RNA detection
(**A**) cDNA from samples A and B derived from human brain tissues was amplified with circular RNA primers circTau exon12_10 Reverse and circTau exon10_11 Forward. The reverse transcription for the cDNA was performed with the primers for linear and circular tau RNA. The expected band corresponding to tau circular RNA is shown by a triangle. The other strong bands are artifacts that did not match the human genome. (**B**) The experiment was repeated with identical PCR conditions, but the reverse transcription was performed only with the circTau exon12_10 Reverse primer. Only the expected band was amplified and validated through sequencing. (**C**) The RNA was treated with RNase R that removes linear RNA. The circular RNA is detectable after the treatment (left), whereas linear RNA gives no longer a detectable signal (right)

Figure 6: Example of an analysis of a circRNA reporter gene
1 µg of the tau 9→12[37] reporter gene was transfected with 1 µg of splicing factors indicated. RNA was isolated 24 h post transfection and analyzed by RT-PCR. (**A**) Amplification of the linear tau mRNA. Due to alternative splicing of exon 10 two bands are observed. Their ratio changes due to the overexpression of splicing factors[38,39]. (**B**) Amplification of the circular 12→10 tau RNA[23]. Note the dependency of tau circRNA expression on expression of some splicing factors, especially the cdc2 like kinase clk2

and the SR protein 9G8. (**C**) The circular RNA of HIPK3 was used as a positive control indicating equal loading.

Table 1: List of current minigenes expressing circular RNAs

Table 2: List of Primers

Supplemental Figure 1: Tau circular RNA test sequence
Test sequence corresponding to a circular RNA from the *MAPT* locus. Different exons are indicated by underline, small caps and large caps.

Supplemental Figure 2: Genomic sequence containing the planned minigene
Exons are highlighted in color and repetitive elements are ***underlined, italic and bold.*** Gray shading indicates flanking regions of low complexity that can be used to generate primers.

Supplemental Figure 3: Sequences of the planned reporter gene
The vector sequence and the planned genomic fragments are shown.

Supplemental Figure 4: Primers design for assembly
The sequence from Supplemental Figure 3 was entered into the builder tool.

Supplemental Figure 5: Sequence of the tau 9->12 reporter gene used as an example.

DISCUSSION:
In general, circular RNAs are low abundant[1], which complicates the study of their function and formation. Similar to linear RNAs[13], the use of reporter minigenes allows the identification of cis and trans-acting factors that regulate the formation of circular RNAs. Thus, this approach generates hypotheses that can be further tested using the endogenous genes.

The most critical step is the design of the reporter gene. The enzymatic assembly of DNA fragments ("Gibson cloning[27]") facilitates this design, as it allows construction of large reporter genes independent of restriction sites.

The back-splicing sites are brought together through flanking inverted repeats, which should be taken into account in reporter gene construction. The repeats are annotated in the genome browser 'repeat track' and selecting them shows their orientation. Keep in mind that proteins can also force the back-splicing sites into a secondary structure needed for circular RNA expression[28] and for an unbiased analysis 1-2 kb of flanking intronic regions should be investigated.

To ensure stability of the constructs, an important consideration is the type of bacterial strains and their growth conditions. For shorter, simple constructs standard cloning bacteria are used, which are almost identical to DH5-alpha (huA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17). For longer fragments, containing more than 6 Alu elements, "stable" competent cells are used that

lack a recombinase (recA) and endonuclease (endA1) (F' proA+B+ lacI^q Δ(lacZ)M15 zzf::Tn10 (Tet^R) Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- Φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (Str^R) rph spoT1 Δ(mrr-hsdRMS-mcrBC). If problems appear with recombination, indicated by low transformation counts, plate the transformed bacteria on two plates and let them grow at 30 ºC and 37 ºC, respectively. Due to the presence of numerous repetitive elements in the minigenes, they need to be fully sequenced using next generation sequencing, which is commercially available for around $150 per plasmid at 2019 rates. The sequence of the example is shown in **Supplemental Figure 5**. In addition, restriction fragment length polymorphism analysis for new larger preparation of the constructs is routinely performed. For example, using sites that cut 1-4 times results in a characteristic band pattern that rules out recombinations (**Figure 4**). Enzymes should be selected that give a characteristic band pattern of fragments that can be separated on an agarose gel.

Circular RNAs are analyzed with RT-PCR using exon junction primers that overlap with the backsplicing event (**Figure 1F**). Due to the circular nature of the RNA, the reverse (i.e., antisense) primer is upstream of the forward (i.e., sense) primer (**Figure 1G**). Primers detecting the abundantly expressed homeodomain-interacting protein kinase 3 (HIPK3) circular RNA[1] are used as a positive control. HIPK3 and minigene specific reverse primers are reverse transcribed in the same tube, which allows their comparison. PCR reactions are performed with primers amplifying the linear mRNAs to compare processing patterns of circular and linear pre-mRNAs. We frequently observed aberrant bands when primers for linear and circular RNAs were mixed (**Figure 5**), and thus keep the reverse transcription of these samples separate.

RT-PCR analysis of circular RNAs is challenging and need to be carefully controlled. While sensitive and convenient, it can produce artifacts unique for circular RNAs[29]. The reverse transcriptase can move several times around the RNA circle, which generates concatemers. Most circular RNA reporter genes generate both circular and linear RNA, which can cross-hybridize, leading to more PCR artifacts[21,30,31]. It is thus imperative to sequence the PCR products and validate findings using different techniques using Northern blots[32] or RNase protections[23].

Unexplained bands can also originate from aberrant amplification of linear RNA. Linear RNA can be removed using the exonuclease RNase R, which enriches circular RNAs[33] (**Figure 5C**). RNase R treatment helps in the initial optimization of detection primers and can often be omitted once primers are optimized.

Alternative back-splicing can also contribute to unexplained bands as multiple circular RNAs can be formed from a genomic locus[34]. This alternative back-splicing is often the result of competing pre-mRNA structures formed by more than two inverted repeat elements. In addition, cryptic back-splice sites can occur[32,35]. Depending on the experimental goal, *Alu*-elements can be repeated or added to the constructs. The complementary regions flanking back-splicing sites can be as short as 30-40 nt[35] and replacement of *Alu* elements with shorter complementary regions can increase circular RNA formation[2], which can be tested to improve circular RNA formation. Once the pre-mRNA sequences that cause back-splicing have been identified, it is thus possible to

shorten circular RNA expressing constructs, which can improve transfection efficiency in some cases.

ACKNOWLEDGMENTS:

This work was supported by the Department of Defense DoD grant AZ180075.

DISCLOSURES:

Nothing to disclose.

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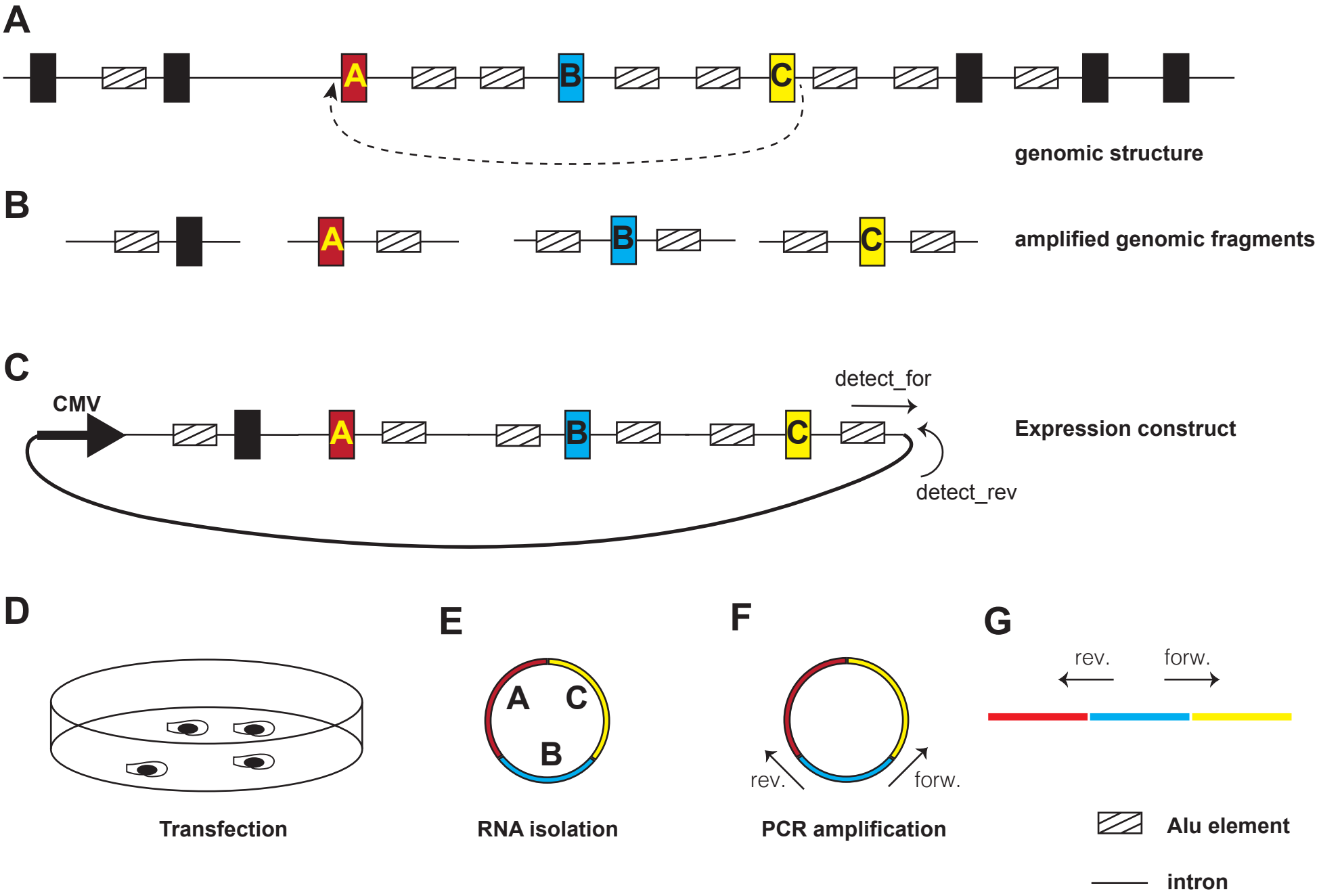

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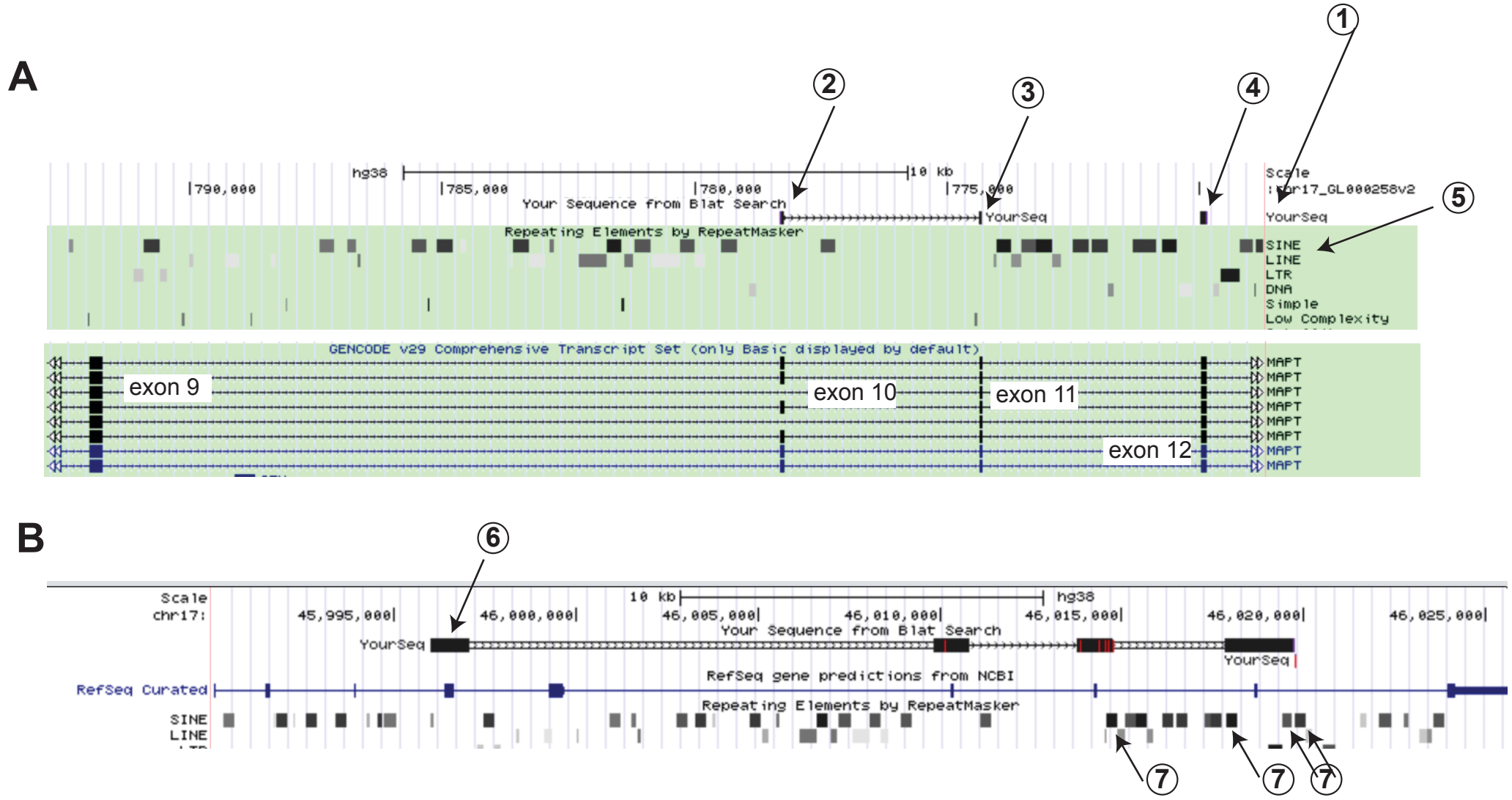

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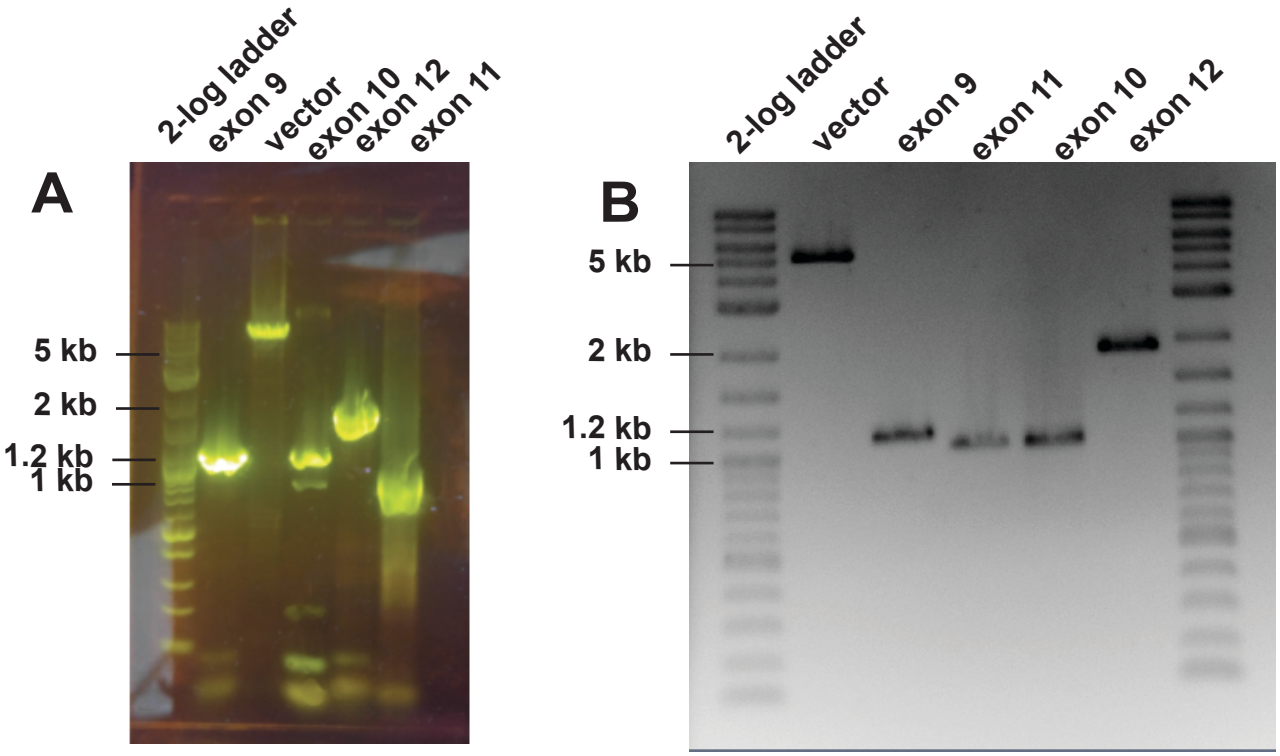

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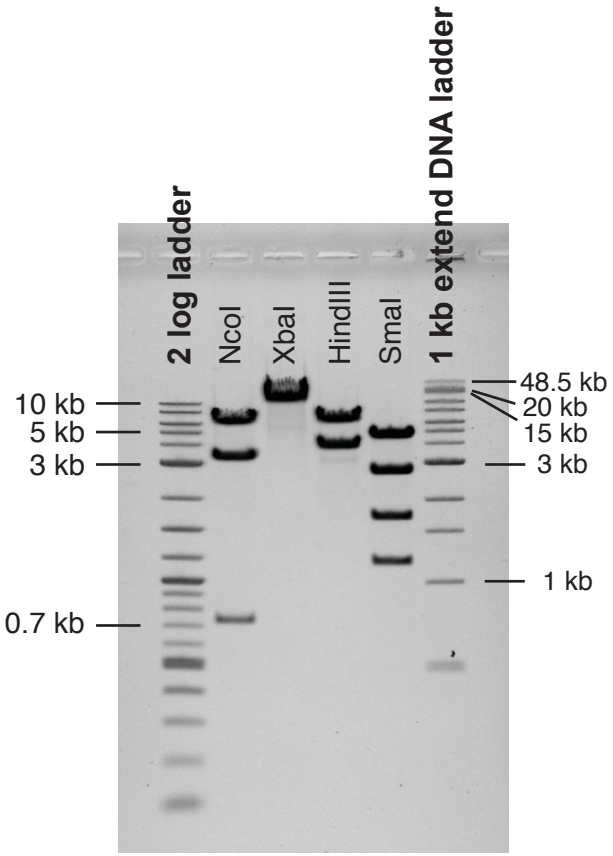

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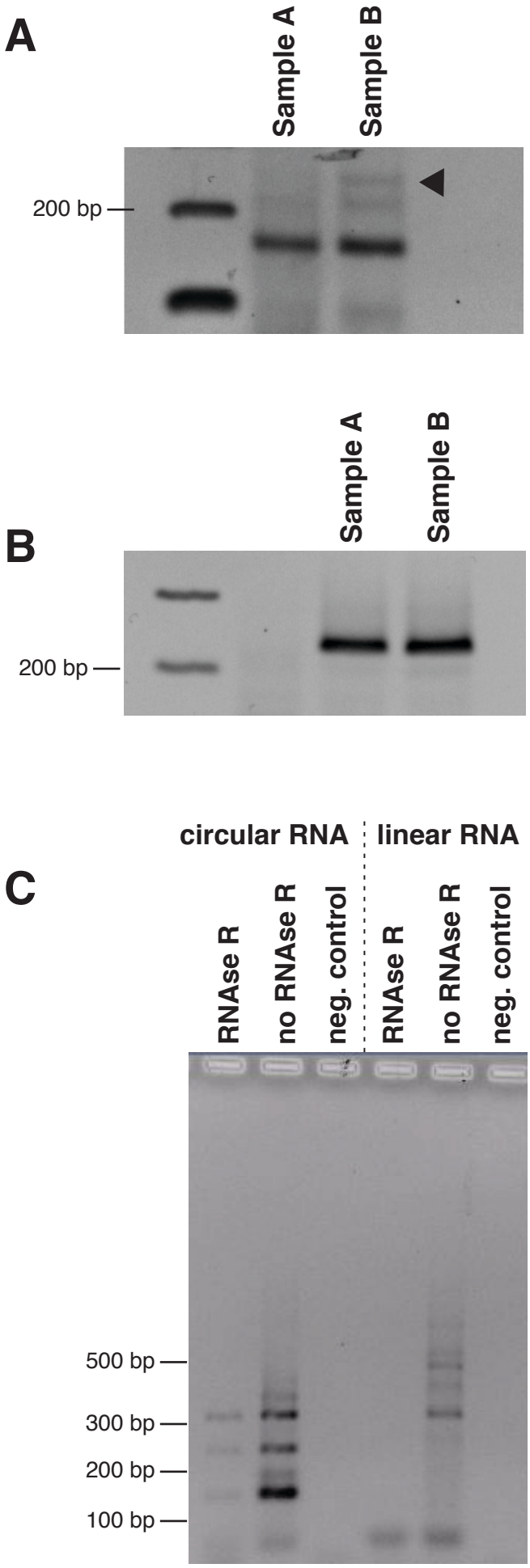


Figure 6

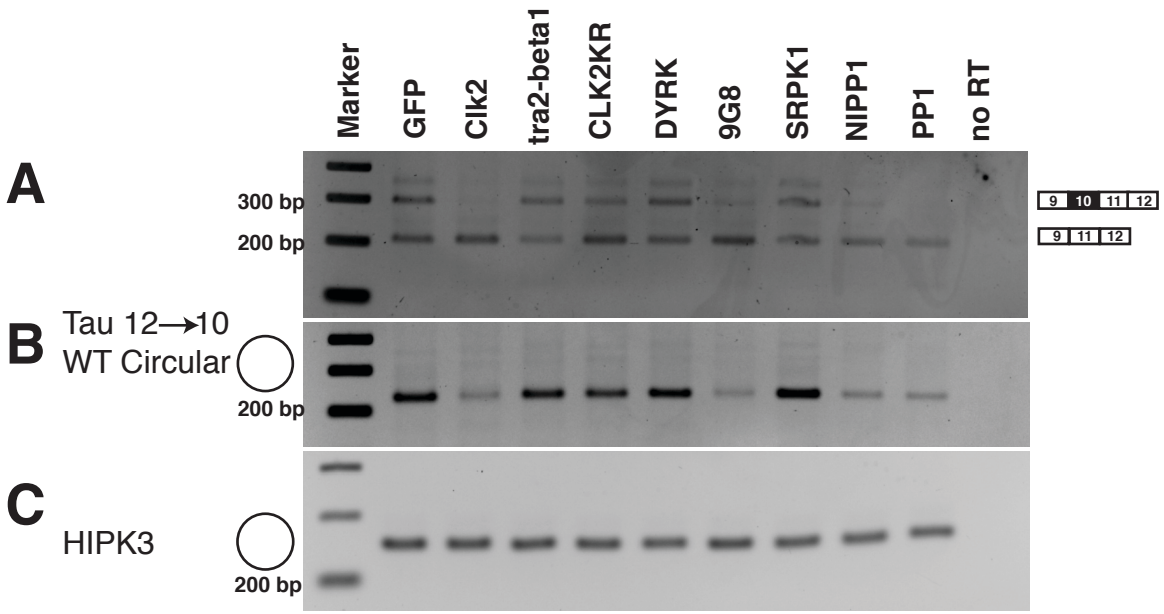
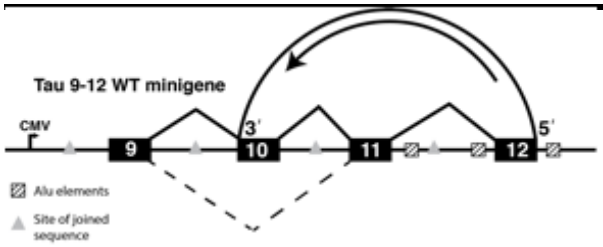
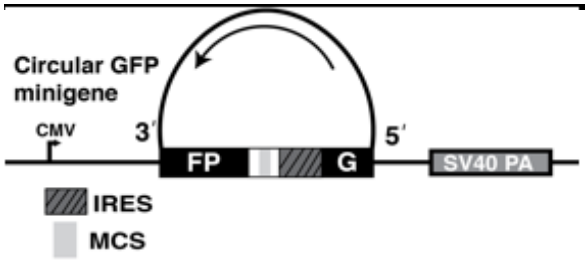
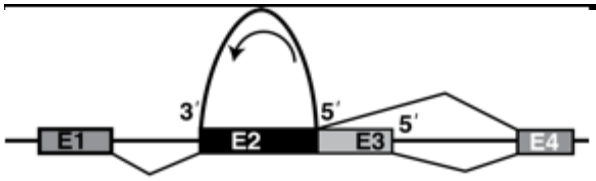
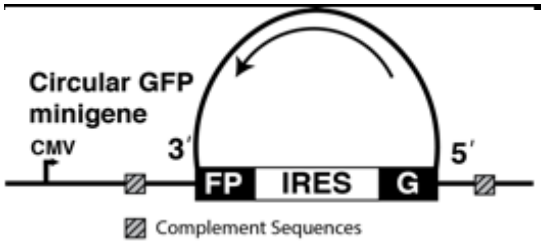


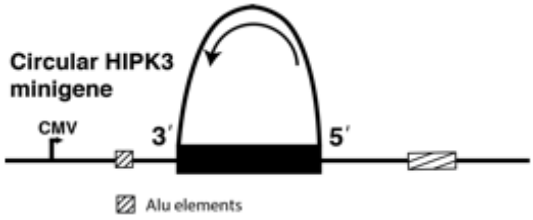
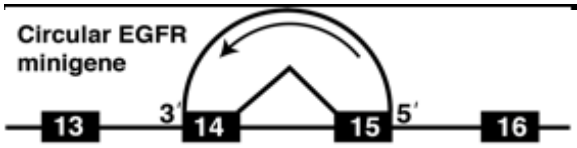
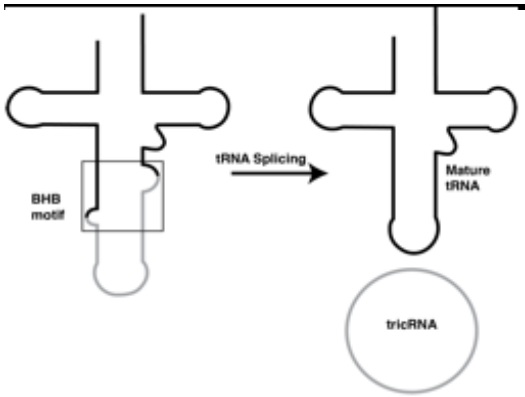
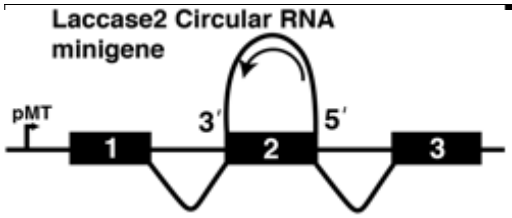
Table 1

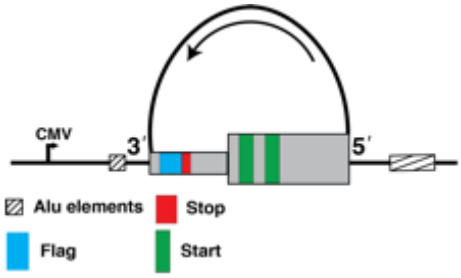
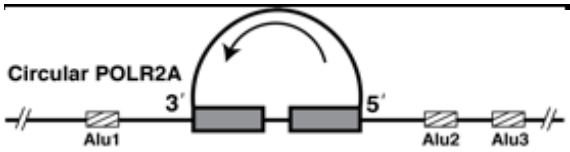
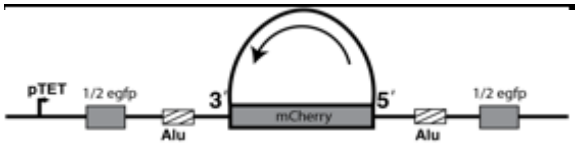
Microtubule-Associated Protein Tau
Circular RNA with 1 Exon (GFP)
circRNA from LPAR1
Circular GFP

Circular HIPK3
epidermal growth factor receptor (EGFR)
circular RNA from tRNAs
Laccase2 CircRNA

CircZNF609
Circular RNA from POL2RA
mCherry circular RNA with IRES
mCherry Circular RNA

 Tau 9-12 WT minigene CMV 3′ 5′ 9 10 11 12 Alu elements Site of joined sequence		23
 Circular GFP minigene CMV 3′ 5′ FP G SV40 PA IRES MCS		40
 3′ 5′ 5′ E1 E2 E3 E4		32
 Circular GFP minigene CMV 3′ 5′ FP IRES G Complement Sequences		41

 Circular HIPK3 minigene CMV 3′ 5′ Alu elements		42
 Circular EGFR minigene 13 3′ 14 15 5′ 16		43
 BHB motif tRNA Splicing Mature tRNA tricRNA		21,22
 Laccase2 Circular RNA minigene pMT 3′ 5′ 1 2 3		44

 CMV 3′ 5′ Alu elements Stop Flag Start		45
 Circular POLR2A 3′ 5′ Alu1 Alu2 Alu3		2,34
 pTET 1/2 egfp 3′ reverted mCherry 5′ 1/2 egfp TAA IRES ATG Alu Alu		46
 pTET 1/2 egfp 3′ 5′ 1/2 egfp mCherry Alu Alu		47

Circular HIPK3 Control Primers	
HIPK3 Reverse	TGCTTGGCTCTACTTTGAGTTTC
HIPK3 Forward	TCGGCCAGTCATGTATCAAA
Linear Primers	
Tau Exon 12 Reverse	CCCAATCTTCGACTGGACTC
Tau Exon 9 Forward	TGTCAAGTCCAAGATCGGCT
Circular Primers	
circTau exon12_10 Reverse	CAGCTTCTTATTAATTATCTGCACCTTTT
circTau exon10_11 Forward	GAGGCGGCAGTGTGCAA

Item	Company / provider
(PEI) Hydrochloride	Polysciences
Builder tool	NEB
Dark Reader Transilluminator.	Clare Chemical Research
Enzymatic DNA assembly kit	NEB
Gel and PCR cleanup kit	Promega
Glyco Blue	Thermo Fisher
pcDNA3.1 cloning site	Polycloning site
Polymerase 1	NEB
Polymerase 2	Biorad
Polymerase 2	Qiagen
Reverse Transcriptase	Thermo Fisher
RNA isolation kit	Life Technologies
RNAse R	Lucigen
Stable competent cells	NEB
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Web tool to design primers	NEB
Web-based temperature calculations	NEB

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Response to Editorial Comments

Editorial comments are in 9 pt font, italic, our response in regular 12 pt font

Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been proofread

2. Authors and affiliations: Please provide an email address for each author.

e-mail contacts have been added

3. Please define acronyms/abbreviations upon first use in the main text.

All acronyms have been defined within the text

4. Summary: Please expand to include a general description of the method and its applications.

We added: These reporter genes are larger than constructs to analyze linear splicing and contain *Alu* elements, which is challenging for cloning. To investigate the circular RNAs, the constructs are transfected into cells and resulting RNA is analyzed using RT-PCR after removal of linear RNA.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please abbreviate liters to L (L, mL, µL) to avoid confusion.

We made these changes accordingly

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Gibson, Q5 Hot Start, Eppendorf Mastercycler, LongAmp, New England Biolabs, Bio-Rad, etc.

Commercial language has been removed. We refer for example to the polymerases as polymerase 1 and 2 and refer them in the tables of Materials. However, to me, this makes the manuscript hard to read

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

The Numbering has been changed

8. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

This has been changed when possible.

9. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

This has been changed into the imperative.

10. Please move the introductory paragraphs of the protocol (lines 65-75, 208-211, 278-279, etc.) to the Introduction, Results, or Discussion (as appropriate).

This paragraph has been removed to the results and discussion section.

11. In the JoVE Protocol format, "NOTE" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Notes has been moved to discussion, there is one Note left in the text.

12. Please specify centrifugation parameters (force in x g and time) throughout the protocol (line 227, etc.).

has been added

13. Please ensure that conditions and primers are listed all PCR procedures.

has been added

14. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

this has been changed for the steps. In some instances there remain longer paragraphs due to explanations

15. Please include single line spacing between each numbered step or note in the protocol.
single line spacing and single paragraphs have been added

16. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

text for videoscript is highlighted

17. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

we highlighted the most important parts

18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

ok

19. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

20. Please number figures/tables in order of their appearance in the text.
ok

21. Figure 3: Please mark the fragment sizes.
markers were added

size markers have been added

22. Table of Materials: Please provide information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please then sort the items in alphabetical order according to the name of material/equipment.
The Table of materials has been made

23. References: Please do not abbreviate journal titles.

We used the JOVE endnote file

We thank all the reviewers for their helpful and insightful comments and revised the paper accordingly.

***Reviewer comment's are in 9 pt font, italic*, our response in regular 12 pt font.**

Reviewer #1:
Manuscript Summary:
The manuscript presents a method to construct minigene generating tau 12->9 circular RNAs. Each procedure is clearly described. Moreover, the authors point out pitfall of each procedure and provide alternative strategies to overcome.

Minor Concerns:
To improve the manuscript, points below need to be addressed .
Page 2 line 50, "is" should be "are".
Page 2 line 86, "Fig.2" should be "Figure 2".
Page 2 line 89, "4" should be "5".
Page 4 line 160, "Note 1" should be bold.
Page 5 line 226, which antibiotic is used?
Page 5 line 227, why are two selection plates needed?
Page 7 line 278, "endonuclease" should be "exonuclease".

All these minor changes have been made

Page 9 line 382, The figure 3A, B needs a size maker with size.

A Size marker was added to Figure 3A and B

Page 9 line 399, It is difficult to compare figure A and B due to cropping the B.

We unified the cropping the cropping

Page 10 line 422, What is highlighted in gray color?

Thanks for spotting this, we added: Gray shading indicates flanking regions of low complexity that can be used to generate primers.

In figure 1A, the arrow needs to move to 3'ss of exon A (red box).

The arrow was moved

In figure 1B, the figure would be easier to read if it shows annealing positions of primers used for amplified genomic fragments.

In order to keep the figure simple, we added to the legend: The genomic fragments will overlap with their flanking 25 nts.

In figure 1E, the figure could show both linear RNAs and circular RNAs. Then, the next figure could show circular RNAs with RNase R treatment.

To keep the figure simple we state : During PCR amplification, linear RNA can also be amplified (G).

In figure 1G, the position of rev. and forw. should be aligned. The yellow color in the exon box could be removed.

The rev. and forw. is now aligned and the yellow exon box was removed

In figure 3, the figure 3A,B needs a size maker with size. The size of exon 11 is different from that of expectation in the figure 3A.

Size markers were added

In figure 6A, diagrammatic representation of splice variants should be given on the right of the gel. It needs a size maker with all the size. It needs one more lane for RNase R treatment.

Diagrammatic representation of splice variants were moved to the right side of the gel and size markers added

We added the effect of RNase R treatment as Figure 5C:

C. The RNA was treated with RNase R that removes linear RNA. The circular RNA is detectable after the treatment (left), whereas linear RNA gives no longer a detectable signal (right)

In figure 6B, it did not match with figure legends. (The circular 12->10 tau RNA.)

Fig. 6B was corrected to 12→10

Other comments:
I would suggest citing this new article describing generation of a huge repertoire of circRNAs from SMN gene. This article describes complementary techniques for characterization of circRNAs.
Ottesen EW, Luo D, Seo J, Singh NN, Singh RN. Human Survival Motor Neuron genes generate a vast repertoire of circular RNAs. Nucleic Acids Res. 2019 Apr 8;47(6):2884-2905.
Thanks for this suggestion, This paper came out during the submission process, we added

Detailed analyses of individual genes using targeted RT-PCR approaches reveal that a large number of circular RNAs remains to be discovered [10]

We thank all the reviewers for their helpful and insightful comments and revised the paper accordingly.

***Reviewer comment's are in 9 pt font, italic*, our response in regular 12 pt font.**

Reviewer #2:

Manuscript Summary:

Paper Welden et al. entitled «Use of Alu-element containing minigenes to analyze circular RNAs» is timely. Authors describe a method to clone and analyze reporter genes generating circular RNAs. Currently, the biogenesis and functional properties of circRNAs remain unclear; transcripts of this class, however, remain highly promising targets of research. There is hypothesis that intronic Alu elements may contribute to alternative splicing (canonic splicing and backsplicing). Undoubtedly, the study of components of Alu elements that are responsible for splicing, and which splicing factors are affected by Alu elements are required. Present work of Welden et al based on their previous study published in Biochim Biophys Acta Mol Basis Dis. 2018. There the authors do not describe in detail the methodological part, which is not trivial. The Alu-element containing expression plasmid constructions to analyze circular RNA were used in studies of Hansen et al, Nature, 2013; Liang and Wilusz, Genes Dev., 2014; Nakama et al, Gene, 2018; and of other teams. This circumstance emphasizes the interest of scientists in this field to such experiments. Methodical article is timely, but revision is required. After this, the manuscript may be acceptable for publication.

Thank you for the insightful comments. We revised accordingly.

Major Concerns:

1. Alu elements occupy 10% of the human genome. Obviously, they form a complex network of interactions in premature RNA. As a result, it contributes to linear RNA, circular RNA, as well as exon-skipping RNA formation. Often Alu repeats belong to different families and may not be highly homologous. Also, it is possible that a few pairs of inverted repeat Alu elements (IRAlus) are involved in splicing. Authors should describe in more detail the principle of choosing a genomic region for a minigene. Also, the Alu elements should be characterized.

We added: The back-splicing sites are brought together through flanking inverted repeats, which should be taken into account in reporter gene construction. The repeats are annotated in the genome browser 'repeat track' and selecting them shows their orientation. It should be kept in mind that proteins can also force the back-splicing sites into a secondary structure needed for circular RNA expression [26] and for an unbiased analysis 1-2 kb of flanking intronic regions should be investigated.

2. For analysis of circRNA using RT-PCR, the authors should clarify, what primers for cDNA synthesis and what ones for PCR were used?

We clarified in the text: We routinely use gene-specific exon-junction primers, but priming with random hexamers is also possible.

3. If authors use minigenes to analyze the participation of splicing factors in circRNA formation, they should pay more attention to choice of splicing factors and description of the results obtained (in Results and Discussion sections). Did the authors use the control without splicing factors? Is it possible to estimate the quantity of factors that entered into the cell of E coli?

Minor Concerns:

1. Video of experiment stages would be very preferable.

this will be done in later stages of the manuscript, following JoVe's procedures

2. *Fig 3 is not very clear. What do the bands mean, and what were the markers used?*

Size markers have been added to the gels. We clarified in the figure legend:

A. Optimized PCR products separated on a 1% agarose gel containing 1x gel green. The individual bands represent the PCR products that will be used enzymatic DNA assembly.
B. The bands from A were cut out from the gel and purified. The purified PCR products were separated on a 1% agarose gel, which was subsequently stained with ethidium bromide.

We thank all the reviewers for their helpful and insightful comments and revised the paper accordingly.

***Reviewer comment's are in 9 pt font, italic*, our response in regular 12 pt font.**

Reviewer #3:
Manuscript Summary:
Dr. Stamm and colleagues present a Alu-element containing minigenes system to study the circRNAs and factors responsible for circRNA biogenesis which is very exciting work.

Minor Concerns:
Having said that, minigene systems might also produce linear and/or circular concatemers (Kramer et al., 2015 G&D, Pamudurti et al., 2017 Mol Cell) I hope the authors aware that. This can be verified by northern blot. although I am convinced by authors RNase R treatment experiment but just to be sure about how efficiently Alu-elements minigenes system generate circRNAs against linear concatemers it would be nicer to have mock vs RNase-R experiment in one panel image. If not at least openly discuss about the linear concatemers in the discussion as caution note of using minigene systems for circRNAs.

Thank you for the comments. We added the necessity about other techniques in the discussion.:

While sensitive and convenient,RT-PCR can produce artifacts unique for circular RNAs [27]. The reverse transcriptase can move several times around the RNA circle, which generates concatemers. Most circular RNA reporter genes generate both circular and linear RNA, which can cross-hybridize, leading to more PCR artifacts [19,28,29]. It is thus imperative to sequence the PCR products and validate findings using different techniques using Northern blots [30] or RNase protections [21]

We also added the effect of RNAse R treatment as Figure 5C:

C. The RNA was treated with RNase R that removes linear RNA. The circular RNA is detectable after the treatment (left), whereas linear RNA gives no longer a detectable signal (right)

We thank all the reviewers for their helpful and insightful comments and revised the paper accordingly.

***Reviewer comment's are in 9 pt font, italic*, our response in regular 12 pt font.**

Reviewer #4:

Manuscript Summary:

In this manuscript, Welden et al describe the details of generation of circular RNAs from Alu-element containing reporter minigenes. Their description is in detail and will be helpful for people who don't have experience in making circular RNA expression vectors. I have the following concerns for the authors to consider to make this protocol more accurate and practical.

Major Concerns:

1*. Table 2 lacks several reported minigenes for circular RNA generation. These include minigenes to generate circular POLR2A with flanking intronic Alus or with flanking intronic complementary sequences (Zhang et al., Cell 2014), the ones for producing alternative circular POLR2A isoforms (Zhang et al., Genome Res 2016); and the dual-color circular mCherry reporter system that was designed for genome-wide trans-factor screening controlled by a pTET promoter (Li et al., Mol Cell 2017). All these reported minigenes not only produce circular RNAs at high yields, but also were developed for different experimental purposes. Therefore, they should all be included in Table 2.*

Three minigenes expressing circular RNAs from Zhang et al., Cell 2014 and Genome Research 2016 and Li et al Mol Cell 2017 have been added to the table.

2. A single gene locus can produce multiple circRNAs via alternative back-splice site selection and/or alternative splice site selection (Zhang et al., Genome Res 2016). Further, it has been shown that minigene vectors can often give rise to cryptic back-splicing products (Starke et al., Cell Rep, 2015; Liang and Wilusz, Genes Dev, 2014). Thus, on one hand, the authors should introduce that minigene vectors can produce different circular RNA isoforms by alternative splicing; on the other hand, the authors should provide suggestions on how to limit unwanted alternatively or cryptically back-spliced circular RNA products.

We added: Alternative back-splicing can also contribute to unexplained bands as multiple circular RNAs can be formed from a genomic locus [32]. This alternative back-splicing is often the result of competing pre-mRNA structures formed by more than two inverted repeat elements. In addition cryptic back-splice sites can occur [30,33]. Depending on the experimental goal, *Alu*-elements can be repeated or added to the constructs. The complementary regions flanking back-splicing sites can be as short as 30-40 nt [33] and replacement of *Alu* elements with shorter complementary regions can increase circular RNA formation [2], which can be tested to improve circular RNA formation.

3. The authors have optimized PCR conditions to generate longer DNA fragments; however, the transfection efficiency can be reduced if the DNA fragment is too large. Alternative suggestions should be made by the authors, such as optimizing and modifying the long flanking introns of circular RNAs. For example, Zhang et al. (Cell 2014) have shown that replacing intronic Alu elements flanking circle-forming exons by shorter but complementary sequences could significantly increase the production of circular RNAs.

We did not really observe changes in transfection efficiency in 293 cells, but this might be different in other cells lines. To address this point, we add:

Once the pre-mRNA sequences that cause back-splicing have been identified, it is thus possible to shorten circular RNA expressing constructs, which can improve transfection efficiency in some cases.

4. In the current manuscript, circular RNAs are analyzed and validated only by RT-PCR using exon junction primers to detect back splicing events. However, it is well-documented that validation circular RNAs by RT-PCR can cause false positive results (Jeck and Sharpless, Nature Biotech 2014), and cryptic back-splicing occurs more frequently in artificial minigene reporters (Starke et al., Cell Rep, 2015; Liang and Wilusz, Genes Dev, 2014). Additional experiments should be carried out, such as Sanger sequencing of individual RT-PCR products and RNase R treatment followed by Northern Blots. More importantly, the Northern Blot analysis will also give rise to the relative back-splicing efficiency could be achieved in such vectors, by detecting both the circular and linear RNA isoforms at the same time. Such information is critical for people who will use the current protocol to generate circular RNAs for Gain-Of-Function assays.
As a protocol manuscript, these additional validations should be important to make the current protocol complete and practical.

We also added the effect of RNAse R treatment as Figure 5C:

C. The RNA was treated with RNase R that removes linear RNA. The circular RNA is detectable after the treatment (left), whereas linear RNA gives no longer a detectable signal (right)
We added to the text:

While sensitive and convenient, it can produce artifacts unique for circular RNAs [27]. The reverse transcriptase can move several times around the RNA circle, which generates concatemers. Most circular RNA reporter genes generate both circular and linear RNA, which can cross-hybridize, leading to more PCR artifacts [19,28,29]. It is thus imperative to sequence the PCR products and validate findings using different techniques using Northern blots [30] or RNase protections [21]

Reviewer #5:

Manuscript Summary:

Welden et al. propose a protocol for cloning and analyzing minigenes for circular RNAs. I read the entire protocol and find it sound, well explained, and appropriately illustrated. I also repeated the first steps in the "Design of the constructs" section. Upon pasting the test sequence into UCSC (as described around lines 81-83), unfortunately I did not get anything that looked like Figure 2A from the top hit. I saw that the window is not green, and the exons are not readily identifiable. But there's probably a minor adjustment of the settings that need to happen that's not currently indicated well enough.

Major Concerns:

Although the Discussion section highlights what could be a significant hurdle (amplifying large amplicons), it would gain in getting expanded, precisely to further illustrate that aspect. At what frequency did the authors observe such recombination events? How do they "validate the reporter plasmids using next generation sequencing"? Some experimental aspects related to detecting recombination are already present at lines 237 and 257, without a clear connection to where such problems would come from, and how big of a problem they represent when carrying out such experiments. Also: What if the recombination event did not affect the restriction sites used in Figure 4? And why this choice of enzymes in particular? It would be worthwhile to contrast Figure 4 to an event in which recombination was observed.

We rechecked Figure 2 and observe the same picture. It is possible that different browser settings give slightly different views.

We observed recombination of clones in about 1 in 15 constructs. However, this frequency depends on the particular gene (for unclear reasons). We therefore do not want to give a ‘firm’ number in the text.

We added:

Due to the presence of numerous repetitive elements in the minigenes, they need to be fully sequenced using next generation sequencing, which is commercially available for around $150 per plasmid at 2019 rates.

Enzymes should be selected that give a characteristic band pattern of fragments that can be separated on an agarose gel.

Minor Concerns:

-To illustrate the importance of sequencing, and that the correct predicted mini-gene was inserted, please show in Supplementary materials the sequencing result (sequence and reads/peaks).

We added Supplemental Figure 5 with the sequence

-Line 39: Citation? Is this predicted from secondary structure analysis or is there experimental evidence for this?

We added these citations (the actual numbers of the citations) are changed in the manuscript:

In humans, *Alu* elements[1], representing about 11 % of the genome [2], form extensive double stranded RNA structures in pre-mRNA due to their self-complementarity [3,4] and thus promote the formation of circRNAs [1].

1 Jeck, W. R. *et al.* Circular RNAs are abundant, conserved, and associated with ALU repeats. *Rna.* **19** (2), 141-157, doi:10.1261/rna.035667.112, (2013).
2 Deininger, P. Alu elements: know the SINEs. *Genome Biol.* **12** (12), 236, doi:10.1186/gb-2011-12-12-236, (2011).
3 Bazak, L., Levanon, E. Y. & Eisenberg, E. Genome-wide analysis of Alu editability. *Nucleic Acids Res.* **42** (11), 6876-6884, doi:10.1093/nar/gku414, (2014).
4 Levanon, E. Y. *et al.* Systematic identification of abundant A-to-I editing sites in the human transcriptome. *Nat Biotechnol.* **22** (8), 1001-1005, doi:10.1038/nbt996, (2004).

-Line 134: The link to the pcDNA3.1 vector does not work.

The URL has been moved to the “table of materials” we checked that the link works by copy pasting into a browser

-Line 272: minor spelling mistake. "sample of the side of the tube" should be "sample off the side of the tube" or "sample from the side of the tube"

This has been changed to sample off the side of the tube

-Figure 1A: Please show the resulting circRNA.

To keep the Figure simple, we only add to the figure legend: The structure of this circular RNA is shown in panel E.

-Figure 1E: Why is the color different? Should keep B blue and C as yellow otherwise it is confusing.

This was an error and has been changed to the same color format.
-Line 392: Missing a paragraph space here.

Has been inserted

-Table 2: tRNA generates circular RNA

The spelling of circular was corrected

Supplemental Figures 1: Test sequence

GTGAACCTCCAAAATCAGGGGATCGCAGCGGCTACAGCAGCCCCGGCTCCCCAGGCACTCCCGGC
AGCCGCTCCCGCACCCCGTCCCTTCCAACCCCACCCACCCGGGAGCCCAAGAAGGTGGCAGTGGT
CCGTACTCCACCCAAGTCGCCGTCTTCCGCCAAGAGCCGCCTGCAGACAGCCCCCGTGCCCATGC
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supplemental figure

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental FIgure 2_rev2.docx

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Exon 9 fragment

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Exon 10 fragment

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Exon 11 fragment

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exon 12 fragment

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New Assembly

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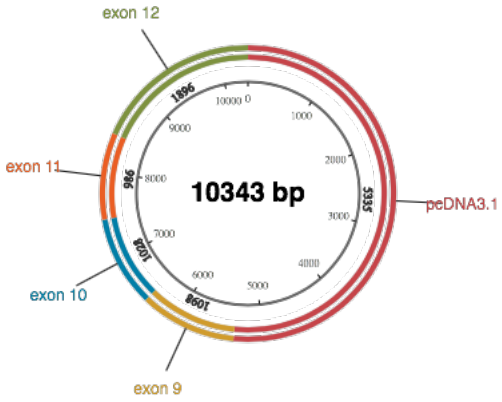
Component Fragments

Name	Length	Produced by	5' End	3' End
pcDNA3.1	5360	PCR	Fwd Primer (auto)	Rev Primer (auto)
exon 9	1124	PCR	Fwd Primer (auto)	Rev Primer (auto)
exon 10	1053	PCR	Fwd Primer (auto)	Rev Primer (auto)
exon 11	1011	PCR	Fwd Primer (auto)	Rev Primer (auto)
exon 12	1920	PCR	Fwd Primer (auto)	Rev Primer (auto)



Notes

- A 60 minute reaction is recommended for the assembly of more than 3 fragments.
- Primer pcDNA3.1_fwd has %GC outside of desired range (35-65%) in the annealing segment.
- Primer exon 9_fwd has %GC outside of desired range (35-65%) in the annealing segment.
- Primer exon 9_rev contains a run of 4+ repeats of a mono/di/trinucleotide.
- Primer exon 9_rev has %GC outside of desired range (35-65%) in the annealing segment.
- Primer exon 10_fwd contains a run of 4+ repeats of a mono/di/trinucleotide.
- Primer exon 10_rev contains a run of 4+ repeats of a mono/di/trinucleotide.
- Primer exon 10_rev has %GC outside of desired range (35-65%) in the annealing segment.
- Primer exon 11_fwd contains a run of 4+ repeats of a mono/di/trinucleotide.
- Primer exon 11_rev contains a run of 4+ repeats of a mono/di/trinucleotide.
- Primer exon 12_fwd contains a run of 4+ repeats of a mono/di/trinucleotide.
- Primers for fragment pcDNA3.1 have internal complementarity.

Required oligos

Name	Primer 5' (overlap/spacer/ANNEAL) 3'	Len	%GC	3' %GC	3' Tm	3' Ta
pcDNA3.1_fwd	gggcccgtttaaa**CCCGCTGATCAGCCTCGA**	31	61	67	70.3	68.4
pcDNA3.1_rev	tgcggccagcgt**AAGCTTAAGTTTAAACGCTAGCCAGC**	38	53	42	67.4	68.4
exon 9_fwd	taaacttaagctt**ACGCTGGCCGCAGGGATT**	31	48	67	73.8	72.0
exon 9_rev	cctgtggatttttt**GGCCCACGAGTGGAGATGC**	32	56	68	71.6	72.0
exon 10_fwd	cactcgtgggcc**AAAAATCCACAGGTGATTCTGATGC**	37	51	40	65.4	66.4
exon 10_rev	tgctggaaaggca**GAGTGGGGTATCTGCGGC**	31	61	67	68.6	66.4
exon 11_fwd	gataccccactc**TGCCTTTCCAGCAAGATTTTTC**	34	47	41	63.6	63.6
exon 11_rev	accatgaccccac**AGTAGCTGGGACTACAGG**	31	58	56	62.6	63.6
exon 12_fwd	gtcccagctact**GTGGGGTCATGGTTTACAG**	31	55	53	62.9	62.0

exon 12_rev	ctgatcagcggg**TTTAAACGGGCCCTCTAG**	30	57	50	61.0	62.0

Build Settings

Property	Value
Product/Kit	#E5520 NEBuilder HiFi DNA Assembly Cloning Kit
Minimum Overlap	25 nt
Minimum Overlap Tm	48 °C
Circularize	Yes
PCR Polymerase/Kit	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Supplemental Figure 5

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