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Isolation and Detection of Telomeric DNA C-Circles from Mammalian Cells

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Abstract:	The identification of cancer cells that promote telomere elongation in the absence of telomerase activity led to the identification of the alternative lengthening of telomeres (ALT) pathway. The ALT pathway is active in approximately 10-15% of all human cancers. However, ALT is most prevalent in some of the most aggressive forms of human cancer including, glioblastoma, osteosarcoma, and pancreatic neuroendocrine tumors. These cancers are highly refractory to common therapeutic strategies and have poor overall survival. Therefore, there has been a growing interest in understanding how, and under what conditions, the ALT pathway is active in an effort to identify therapies that uniquely target the ALT mechanism. These efforts necessitate the use of a robust biomarker to not only identify ALT positive cancers, but also to monitor ALT activity throughout treatment. Several cellular phenotypes have been identified and demonstrated to correlate with ALT activity including the production of extrachromosomal telomeric repeat (ECTR) DNA. ECTR exist in both linear and circular forms containing either C-rich or G-rich partially double-stranded telomeric sequences. To date, the circular C-rich telomeric sequences (C-circles) are the only ECTR DNA products that have been demonstrated to be exclusive to ALT positive cancer cells. In this protocol, we demonstrate a technique used to isolate and detect C-circles from mammalian cells highlighting the utility of this assay in the determination of ALT status.
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Dear Editorial Board,

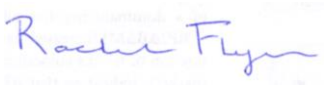
I would like to submit the revised manuscript entitled "Isolation and Detection of Telomeric DNA C-Circles from Mammalian Cells" for publication in the Journal of Visualized Experiments. In this manuscript we describe a technique used to determine whether a cancer cell relies on the Alternative Lengthening of Telomere pathway to promote telomere elongation.

The identification of cancer cells that promote telomere elongation in the absence of telomerase activity led to the identification of the alternative lengthening of telomeres (ALT) pathway. The ALT pathway is active in approximately 10-15% of all human cancers. However, ALT is most prevalent in some of the most aggressive forms of human cancer including, glioblastoma, osteosarcoma, and pancreatic neuroendocrine tumors. These cancers are highly refractory to common therapeutic strategies and have poor overall survival. Therefore, there has been a growing interest in understanding how, and under what conditions, the ALT pathway is active in an effort to identify therapies that uniquely target the ALT mechanism. These efforts necessitate the use of a robust biomarker to not only identify ALT positive cancers, but also to monitor ALT activity throughout treatment. Several cellular phenotypes have been identified and demonstrated to correlate with ALT activity including the production of extrachromosomal telomeric repeat (ECTR) DNA. ECTR exist in both linear and circular forms containing either C-rich or G-rich partially double-stranded telomeric sequences. To date, the circular C-rich telomeric sequences (C-circles) are the only ECTR DNA products that have been demonstrated to be exclusive to ALT positive cancer cells. In this protocol, we demonstrate a technique used to isolate and detect C-circles from mammalian cells highlighting the utility of this assay in the determination of ALT status.

Author Contribution: Emily Mason-Osann and Kelli E. Cox performed and analyzed the experiments. Emily Mason-Osann, Kelli E. Cox, and Rachel Litman Flynn wrote the manuscript

Please feel free to contact me if any additional information can assist you in evaluating this revised manuscript. Thank you again for your consideration.

Sincerely,



Rachel L Flynn

TITLE:
Isolation and Detection of Telomeric DNA C-Circles from Mammalian Cells

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SHORT ABSTRACT:
Cancer cells can overcome replicative senescence by activating the alternative lengthening of telomeres (ALT) pathway. ALT positive cancer cells are uniquely characterized by the production of circular C-rich telomeric DNA sequences (C-circle). This protocol describes how to detect C-circles isolated from ALT-positive mammalian cells.

LONG ABSTRACT:
The identification of cancer cells that promote telomere elongation in the absence of telomerase activity led to the identification of the alternative lengthening of telomeres (ALT) pathway. The ALT pathway is active in approximately 10-15% of all human cancers. However, ALT is most prevalent in some of the most aggressive forms of human cancer including, glioblastoma, osteosarcoma, and pancreatic neuroendocrine tumors. These cancers are highly refractory to common therapeutic strategies and have poor overall survival. Therefore, there has been a growing interest in understanding how, and under what conditions, the ALT pathway is active in an effort to identify therapies that uniquely target the ALT mechanism. These efforts necessitate

the use of a robust biomarker to not only identify ALT positive cancers, but also to monitor ALT activity throughout treatment. Several cellular phenotypes have been identified and demonstrated to correlate with ALT activity including the production of extrachromosomal telomeric repeat (ECTR) DNA. ECTR exist in both linear and circular forms containing either C-rich or G-rich partially double-stranded telomeric sequences. To date, the circular C-rich telomeric sequences (C-circles) are the only ECTR DNA products that have been demonstrated to be exclusive to ALT positive cancer cells. In this protocol, we demonstrate a technique used to isolate and detect C-circles from mammalian cells highlighting the utility of this assay in the determination of ALT status.

INTRODUCTION:

Telomeres are repetitive DNA sequences that cap the ends of linear chromosomes to protect chromosome ends from degradation and end-to-end fusions. Given the semiconservative nature of DNA replication telomeres shorten with each successive cell division. Eventually, telomeres reach a critically short length and induce cellular senescence and crisis. Cancer cells must overcome telomere shortening to bypass cellular senescence and gain replicative immortality.[1] Approximately 85% of cancers promote telomere elongation by reactivating the enzyme telomerase.[2] Another 10% of cancers activate a second mechanism called the Alternative Lengthening of Telomeres (ALT) pathway.[2,3] While both pathways promote telomere elongation, and ultimately cellular immortality, the mechanism(s) for reactivation and subsequent elongation have not been fully elucidated.

Telomere maintenance mechanisms are an attractive target in the treatment of cancer as these therapies limit the replicative potential of cancer cells while largely sparing normal somatic cells.[4] While telomerase inhibitors have entered into clinical trials, cancer cells that rely on ALT will not be sensitive to telomerase-targeted therapies.[4] Moreover, ALT activation may provide a mechanism of resistance following telomerase inhibition[5]. Recent studies have demonstrated that ALT positive cancers are sensitive to inhibition of the DNA damage response kinase, Ataxia Telangiectasia and Rad3 related (ATR),[6] highlighting the potential for an ALT targeted therapy in the treatment of cancer.

Cancer cells with an active ALT mechanism are characterized by a number of unique cellular phenotypes. These phenotypes have not only served as surrogates for ALT activity, but have also been instrumental in developing our understanding of the ALT mechanism. One of the first observations made in ALT cells was that the telomeric DNA was incredibly dynamic, undergoing rapid increases and decreases in mean telomere length[3]. This extreme heterogeneity in telomere length was one of the first indications that telomeres in ALT cells may use recombination to promote telomere elongation. This was later supported by studies demonstrating that a single DNA tag integrated into the telomeric region on one chromosome was copied to the telomeres of neighboring chromosomes[7]. This heightened frequency of recombination specifically at the telomeres is now defined as an increase in telomere sister chromatid exchange (T-SCE)[8]. ALT telomeres are also recruited into nuclear foci that, in addition to the promyelocytic leukemia (PML) protein, contain a number of recombination and repair factors[9]. These ALT-associated PML bodies (APB) associate with the extrachromosomal telomeric repeat (ECTR) DNA generated in ALT cells. ECTR DNA exists in both linear and circular forms and while it is likely generated as a byproduct of homologous recombination

(HR), it might also perpetuate the ALT phenotype by functioning as a template for elongation[10,11]. Together, these cellular phenotypes have highlighted the importance of recombination for telomere elongation in the ALT pathway.

The formation of APBs and frequency of T-SCE have been used as surrogate markers for ALT activity. However, both APB and T-SCE can be found in non-ALT cells[12] and in contrast, some ALT cells do not have either APB or T-SCE.[13] Additionally, measuring these phenotypes has limitations. Measuring T-SCE requires the use of the chromosome orientation fluorescence *in situ* hybridization (Co-FISH) assay to analyze recombination events at individual telomere ends of metaphase spreads. These assays can be technically challenging and require the use of proliferating cells which are not often available with clinical samples. While APBs can be measured by combined immunofluorescence and in situ hybridization on fixed samples, APBs are sometimes found in as few as 0.5% of cells in an ALT tumor sample which may limit the reliability of this assay as a robust diagnostic marker.[14,15]

Extrachromosomal telomeric repeats exist in several forms, including C-rich circularized DNA, or C-circles.[11,16] C-circles are a circular, partially single-stranded, C-rich telomeric sequence (CCCTAA) with a partial double-stranded region containing the G-rich complementary strand (TTAGGG). C-circles are not only likely to contribute to the ALT mechanism, but they also serve as a robust readout of ALT status[11,17]. Given the prevalence of C-circles in ALT cells, Henson *et al.* developed the C-circle assay to measure ALT activity in cell lines and/or patient blood samples. This assay relies on the ability of the C-circle to prime itself using the partial G-strand sequence to initiate rolling circle amplification (RCA), allowing detection of a robust signal from a small amount of genomic DNA.[17] While we will describe how to measure C-circles from genomic DNA isolated from cell lines in culture, this protocol has also been used to measure C-circles from blood samples of patients with ALT tumors.[17] In addition to being both highly sensitive and predictive for ALT status, the C-circle protocol relies on the use of common laboratory equipment, making it suitable for most standard biochemistry, cellular, and/or molecular biology laboratories.

PROTOCOL:

1. Isolate genomic DNA

1.1. Collect cells from a 10 cm dish with trypsin or by scraping and collect in a microcentrifuge tube.

1.1.1. Wash cells with 3-5 ml of PBS.

1.1.2. If collecting cells by scraping, scrape dish and collect. Otherwise, aspirate PBS from dish.

1.1.3. Add 1.5 ml of 0.05% trypsin-EDTA and incubate at 37 °C for 3-5 min or until cells detach from plate.

1.1.4. Add 5-10 ml of media containing 10% fetal bovine serum and wash the plate.

Note: This step is only necessary if cells are adherent, for suspension cells step 1.1 can be skipped.

1.2. Centrifuge the cell suspension at 300 x g for 5 min at room temperature to pellet the cells and then aspirate the media without disturbing the pellet.

1.3. Resuspend the cell pellet in 1 ml of 1X PBS.

1.4. Centrifuge the cell suspension at 300 x g for 5 min at room temperature to pellet the cells and then aspirate the PBS without disturbing the pellet.

1.5. Isolate the genomic DNA from the cell pellet using a DNA isolation kit, following the manufacturer's instructions for mammalian cells. Elute the DNA into a microcentrifuge tube with 80 µL ddH_2O.

1.6. Measure the genomic DNA concentration using a microvolume spectrophotometer.

2. Digest genomic DNA

2.1. Make a restriction digestion master mix with 10 µl 10X restriction enzyme buffer, 5 µl Alu1 (50 Units), 5 µl Mbo1 (25 Units), and 0.2 µl of 1 mg/ml RNase A for each sample.

Note: 10X restriction enzyme buffer is normally supplied with restriction enzymes. It contains 500 mM potassium acetate, 200 mM tris-acetate, 100 mM magnesium acetate, and 1 mg/ml bovine serum albumin at pH 7.9.

2.2. To digest 15 µg of genomic DNA for each sample (more than 15 µg of DNA will decrease the efficiency of the digest), mix 15 µg of DNA with 20.2 µl of master mix. Bring the total volume of each reaction up to 100 µl with ddH_2O and mix thoroughly. Incubate the reaction at 37 °C overnight.

2.3. Following the overnight incubation, remove the restriction enzymes from the digested genomic DNA using a PCR purification kit, following the manufacturer's instructions.

Note: Following restriction enzyme digestion, verify the efficiency of the digestion by running a small sample of the digested genomic DNA on a 1% agarose gel.

2.4. Measure the DNA concentration using a microvolume spectrophotometer.

3. C-circle amplification from digested genomic DNA

3.1. Dilute digested genomic DNA to 10.0 ng/µl in ddH_2O to a volume of 250-400 µl.

3.2. Measure the DNA concentration using a microvolume spectrophotometer. Adjust concentration as necessary until 3 consecutive readings achieve 10.0 ng/µL. This step is crucial to reduce variations between samples.

3.3. Prepare a master mix for the amplification reaction such that each reaction contains 10 µl of 0.2 mg/ml bovine serum albumin (BSA), 2.5 µl of 10X Phi 29 buffer, 0.25 µl of 10% Tween-20, and 0.25 µl of 25 mM deoxynucleotides (dTTP, dGTP, dATP). Prepare enough master mix for 4 amplification reactions for each sample. Add 13 µl of master mix to each tube.

Note: Omitting dCTP ensures specific amplification of the G-strand of C-circles, which contains only TTAGGG repeats and does not require dCTP.

3.4. Prepare an amplification reaction using 10 ng, 20 ng, and 40 ng of DNA for each sample by adding 1 µl, 2 µl or 4 µl of DNA prepared in 3.2, respectively, to the 13 µl of master mix. Prepare one additional reaction containing 40 ng of DNA. This reaction will not receive the polymerase and will serve as a negative control for the amplification reaction.

3.5. Add 0.75 µl of the Phi29 DNA polymerase to each reaction tube except for the negative control. Bring the total reaction volume up to 25 µl using ddH_2O.

3.6. To amplify C-circles, incubate the reaction at 30 °C for 8 h. Following the amplification, inactivate the polymerase by increasing the temperature to 65 °C and incubating the reaction for 20 min.

Note: This can be done in a water bath or a thermocycler. When using a thermocycler, this reaction can be run overnight, products remain stable if held at 4 °C following the amplification.

4. Analyze C-circle amplification products using a DNA dot blot

4.1. Prepare 10X SSC buffer.

4.1.1. Prepare a 1 L stock of 20X SSC by adding 175.3 g sodium chloride and 88.2 g sodium citrate to ddH_2O. Bring total volume to 1 L with ddH_2O.

4.1.2. Dilute 20X SSC by mixing equal volumes 20X SSC and ddH_2O.

4.2. Cut two pieces of chromatography filter paper and a single piece of positively charged nylon membrane to fit dot blot apparatus (approximately 11.3 cm x 7.7 cm). Clip one corner of the membrane to mark the position of the membrane during sample loading.

4.3. Soak the filter papers and nylon membrane in 10X SSC for 10 min at room temperature.

4.4. Warm the nucleic acid hybridization buffer to 65 °C in a water bath.

4.5. Dilute the amplification reaction products from step 3.6 to 200 µL using 10X SSC.

4.6. Set up the dot blot apparatus.

4.6.1. Place two pieces of filter paper on top of the rubber gasket.

4.6.2. Place the membrane directly on top of the filter papers, with the clipped corner lining up with the lower right corner of the dot blot apparatus.

4.6.3. Place the top of the dot blot apparatus over the membrane and tighten the screws by hand. Ensure that the screws are tightened evenly to avoid leakage.

4.6.4. Fill each well with 200 µl of 10X SSC using a multichannel pipette.

4.6.5. Turn on the vacuum to pull the 10X SSC through the membrane.

4.6.6. Tighten the screws while the vacuum is on to ensure a tight seal (do not over tighten).

4.6.7. Turn off the vacuum.

4.7. Fill all of the wells with 10X SSC except for the wells that will be used for the samples.

Note: Samples can be arranged in any orientation, though it is best to avoid using the outermost wells.

4.8. Load 200 µl (full volume) of samples, each into an individual well in the dot blot apparatus. Be careful to avoid introducing air bubbles into the sample wells, as it will interfere with the ability of the vacuum to pull the sample onto the membrane.

4.9. Turn on the vacuum and watch for samples to be pulled through membrane.

4.10. Turn off the vacuum as soon as the last sample is pulled through the membrane, all the wells in the dot blot apparatus should be empty.

4.11. Remove the top of the dot blot apparatus and use forceps to peel the membrane off of the filter papers.

4.12. Place the membrane on a clean flat surface, with the side containing the sample facing up.

4.13. Crosslink the membrane for 35 s in a UV crosslinker (181,540 µJ).

4.14. Place the crosslinked membrane inside of a hybridization bag and heat seal the bag on three sides using an impulse heat sealer.

4.15. Add 8 ml of pre-warmed nucleic acid hybridization buffer to the hybridization bag and heat seal the final side using an impulse heat sealer.

4.16. Incubate the blot in hybridization buffer for 30-60 min at 50 °C in a hybridization oven. Either rock the blot gently on a shaking platform or attach the blot to a rotisserie and rotate the blot during the incubation.

Note: A rotary bottle can be used for hybridization instead of a hybridization bag, however, it requires higher volumes of hybridization buffer.

5. Probe dot blot for telomeric sequence

5.1. To detect the C-circle amplification products, label the telomeric $(CCCTAA)_4$ antisense oligonucleotide with digoxigenin following the manufacturer's instructions.

5.1.1. Mix 100 pmoles of the antisense telomeric oligonucleotide (4 µl of 25 µM stock; 5' – CCCTAACCCTAACCCTAACCCTAA – 3'), 4 µl of 5X reaction buffer, 4 µl of $CoCl_2$ solution (25 mM stock), 1 µl of DIG-ddUTP solution (1 mM stock) and 1 µl recombinant terminal transferase (400 U/µl stock). Bring the total reaction volume up to 20 µl by adding 6 µl of ddH_2O.

5.1.2. Mix the labeling reaction thoroughly and incubate in a water bath or a heat block at 37 °C for 30-60 min. Remove the labeling reaction from the water bath and stop the reaction by adding 1 µl of 0.5M EDTA pH 8.0. Keep the labeled probe on ice.

Note: The labeled probe can be stored at -20 °C and reused for up to a week, longer store times have not been tested.

5.2. Add 10 µl of the labeled probe (from step 5.1) to 4 ml of fresh hybridization buffer and mix thoroughly with a pipette.

5.3. Remove the blot from the hybridization oven and cut one side of the hybridization bag open. Add the 4 ml of fresh hybridization buffer that contains the labeled telomeric probe to the 8 ml of hybridization buffer already in the hybridization bag. Mix the hybridization buffer gently inside the bag using a pipette, but avoid introducing excessive air bubbles.

5.4. Gently remove any bubbles from the hybridization bag and reseal the fourth side of the hybridization bag using a heat sealer.

5.5. Incubate the membrane overnight at 50 °C in the hybridization oven. Either rock the blot gently on a shaking platform or attach the blot to a rotisserie and rotate the blot during the incubation period.

5.6. Prepare wash buffers and detection buffers.

5.6.1. Prepare 2X SSC with 0.1% SDS.

5.6.2. Prepare 0.5X SSC with 0.1% SDS.

5.6.2.1. Warm the 0.5X SSC with 0.1% SDS buffer to 50 °C in a water bath.

5.6.3. Prepare the 10X wash buffer comprised of 1 M maleic acid, 1.5 M NaCl, and 0.3% Tween-20 at pH 7.5.

5.6.4. Prepare the 10X maleic acid buffer comprised of 1 M maleic acid and 1.5 M NaCl at pH 7.5.

5.6.5. Prepare the 10X blocking buffer comprised of 10% blocking reagent in 1X maleic acid buffer (prepared from 10X maleic acid buffer diluted in ddH_2O).

Note: The 10X blocking buffer should be opaque.

5.6.5.1. To get the blocking reagent into solution, heat the buffer and stir extensively. Once most of the blocking reagent is in solution, autoclave the buffer for 30 min.

Note: This buffer can be made ahead of time, aliquoted and stored at -20 °C.

5.6.6. Prepare the 10X detection buffer comprised of 1 M Tris-HCl and 1 M NaCl at pH 9.5.

Note: All buffers are diluted from concentrated stocks to working concentrations with ddH_2O unless otherwise stated.

5.7. Remove the membrane from the hybridization bag and place the membrane in a clean container that is slightly larger than the membrane.

5.8. Add 30-40 ml of 2X SSC + 0.1% SDS to the container and wash 2 x 5 min at room temperature while gently shaking. Refresh buffer between washes.

5.9. Remove the membrane from the container and place the membrane into a glass hybridization bottle.

5.10. Add 30-40 ml of 0.5X SSC + 0.1% SDS to the bottle and wash 2 x 15 min at 50 °C in the hybridization oven. Place glass bottle in rotisserie in hybridization oven to rotate during washes. Refresh buffer between washes.

5.11. Remove the membrane from the glass hybridization bottle and place the membrane in a clean container.

5.12. Add 30-40 ml of 1X wash buffer (prepared from 10X stock made in step 5.6.3) to a clean container and wash the membrane at room temperature for 1-5 min while gently shaking.

5.13. Prepare the 1X blocking buffer by diluting 10X blocking buffer in 1X maleic acid buffer (5 ml of 10X blocking buffer, 5 ml of 10X maleic acid buffer and 40 ml of ddH_2O).

5.14. Place the membrane in a clean container and add 25 ml of 1X blocking buffer. Incubate at room temperature for 30 min while gently shaking.

5.15. Dilute the anti-digoxigenin Fab fragment conjugated to alkaline phosphatase (anti-DIG-AP) 1:10,000 in 1X blocking buffer (2.5 µl in 25 ml of 1X blocking buffer).

5.16. Incubate the membrane with the anti-DIG-AP in blocking buffer for 30 min at room temperature while gently shaking.

5.17. Place the membrane in a clean container. Wash the membrane with 30-40 ml 1X wash buffer (diluted from 10X stock made in step 5.6.3) 2 x 15 min at room temperature while gently shaking. Place the membrane in a clean container and refresh buffer between washes.

5.18. Place the membrane on a clean flat surface and equilibrate the membrane in 9 ml of 1X detection buffer.

5.19. Dilute the alkaline phosphatase chemiluminescent substrate to 0.25 mM in 1 ml of detection buffer.

5.20. Incubate the membrane in chemiluminescent substrate solution for 5 min at room temperature. Image the membrane with a chemiluminescence imager to detect signal. The membrane may need to be exposed for up to 60 min.

5.21. Quantify the signal using densitometry and determine fold change by normalizing to a control sample.

REPRESENTATIVE RESULTS:

Cells that rely on the ALT pathway for telomere maintenance are characterized by a number of cellular phenotypes, including the formation of partially single-stranded circular C-rich telomeric DNA. C-circles are unique to ALT cells and can be detected using rolling circle amplification of genomic DNA isolated from mammalian cells as depicted in Figure 1. Figure 2A is a representative experiment demonstrating the detection C-circle products in the ALT positive U2OS and telomerase positive SJSA1 osteosarcoma cell lines by DNA dot blot. Increasing the concentration of genomic DNA from each cell line added to the initial RCA reaction demonstrates an increase in the abundance of C-circle products only in ALT positive cells. The relative abundance of C-circle products can be quantified using densitometry as demonstrated in Figure 2B. These data demonstrate that the genomic DNA isolated from U2OS cancer cells, and not the SJSA1 cancer cells, contains C-circles confirming that the U2OS cell line is ALT positive.

Figure Legends

Figure 1: Overview of C-circle assay protocol. First, genomic DNA is isolated from human cancer cells. Second, the genomic DNA is digested using restriction enzymes that do not cleave the telomeric DNA, leaving C-circles undigested. Third, C-circles are amplified in an isothermic rolling circle amplification reaction using the phi29 DNA polymerase. No primers are added to the amplification because the C-circles autoprime using the partial G-rich strand. Last, the amplification products are crosslinked to a membrane and detected with digoxigenin labeled $(CCCTAA)_4$ probe. The digoxigenin can be detected by chemiluminsecence using an enzyme linked anti-DIG antibody.

Figure 2: Representative results of a C-circle assay for ALT and non-ALT cell lines. A) DNA dot blot is used to detect amplification products of the C-circle assay. Results shown are products of amplification of 10, 20 or 40 ng of genomic DNA starting material. The no polymerase control contains genomic DNA and all components of the amplification reaction except for phi29 polymerase. U2OS is an ALT cell line, and SJSA1 is a non-ALT cell line that uses telomerase for telomere maintenance. B) The amplification products from 40 ng of genomic DNA were quantified using densitometry. Values were adjusted for background and normalized to SJSA1 to determine relative C-circle abundance. (Mean±SD, n=3)

DISCUSSION:

The identification of cancer cells that maintain telomere length for over 100 population doublings in culture, in the absence of telomerase activity, led to the identification of the ALT pathway[3]. Since that initial discovery twenty years ago there has been a growing interest in

defining mechanistically how, and under what conditions, the ALT pathway is active in cancer. To date, the literature suggests that the ALT pathway is active in approximately 10-15% of all human cancers, with the highest prevalence in osteosarcoma, glioblastoma, and pancreatic neuroendocrine tumors[15,18,19]. These cancers are highly refractory to common therapeutic strategies and have poor overall survival highlighting the need for both robust clinical biomarkers and improved therapeutic strategies for patients. Although a number of cellular phenotypes have been described to correlate with ALT status, only the C-circle Assay provides the specificity and reliability critical for utility as a clinical diagnostic.

The C-circle assay relies on the amplification of C-circle products present in the genomic DNA of human samples using rolling circle amplification[17]. Rolling circle amplification (RCA) is an *in vitro* biochemical reaction that uses unidirectional DNA replication to generate multiple copies of a circular nucleic acid. One limitation of the C-circle assay is that there is not an internal control for the RCA reaction itself and consequently, no effective way to normalize the efficiency of the reactions from sample to sample. This can make comparing changes in C-circle abundance across many samples technically challenging. As a result, this may limit the usefulness of this assay in analyzing specific changes in ALT activity in high throughput drug and/or genomic screens. However, this limitation should not take away from the fact that the C-circle assay is the most robust assay available for the determination of ALT status.

The RCA reaction can amplify C-circles present in as little as 7.5 nanograms of genomic DNA making the C-circle assay advantageous over other ALT assays including APB and T-SCE. Given that tumor derived DNA is found in the blood of cancer patients,[20] the C-circle assay has the ability to detect ALT from blood samples, unlike APB or T-SCE. We have chosen to isolate and purify genomic DNA using commercially available purification kits to streamline the assay. However, if cost is a concern, genomic DNA can also be easily isolated using standard laboratory reagents and supplies. Likewise, once the genomic DNA has been digested with the indicated restriction enzymes the remaining DNA can be isolated using ethanol precipitation. Finally, while the linear range of detection for C-circles is achieved using between 10 and 30 nanograms of genomic DNA in the initial RCA reaction, it can be difficult to obtain consistent and accurate readings at these low DNA concentrations across many samples using a traditional spectrophotometer. Therefore, the use of a DNA fluorometer can be implemented to improve the accuracy of DNA quantifications.[17] This is a useful modification to the protocol that we have not demonstrated here, but requires nontraditional equipment and/or reagents.

The protocol described here relies on the use of digoxigenin labeled probes to detect the final C-circle amplification products by chemiluminescence. However, digoxigenin based detection methods can present several challenges for researchers including the cost of reagents and the reliance on alkaline phosphatase chemiluminescence which may not be optimum for quantification. Alternatively, DNA dot blots can also be analyzed using radiolabeled probes or fluorochrome-conjugated probes. Although these detection methods may present with their own sets of challenges, it is important to note that alternative methodologies exist.

There is a growing interest in the development of therapies that uniquely target mechanisms that promote telomere elongation. Robust assays to analyze ALT, like the C-circle assay, will be essential for not only defining the mechanism(s) regulating the ALT pathway, but also

facilitating the development of targeted therapies. Identifying patients that may benefit from these targeted therapies would improve treatment and undoubtedly patient outcomes. Here, we have demonstrated how to analyze C-circles in human ALT positive cancer cell lines. However, C-circles can be isolated and detected in the genomic DNA isolated from both tumor tissue and blood samples. The identification and characterization of C-circles, and the development of an assay to detect these DNA elements has provided valuable insight in the field and provided the framework for future advances in both basic and translational research.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

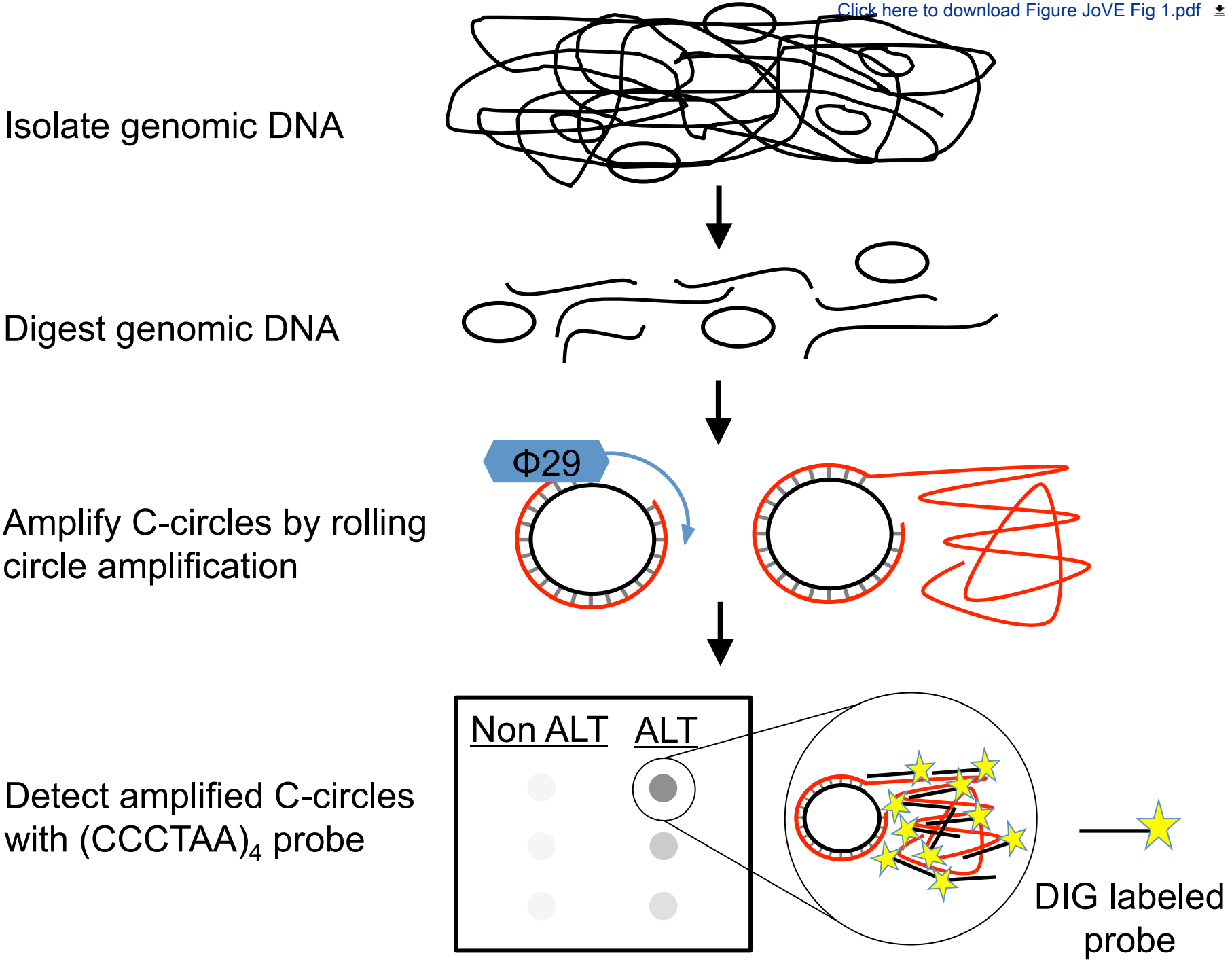
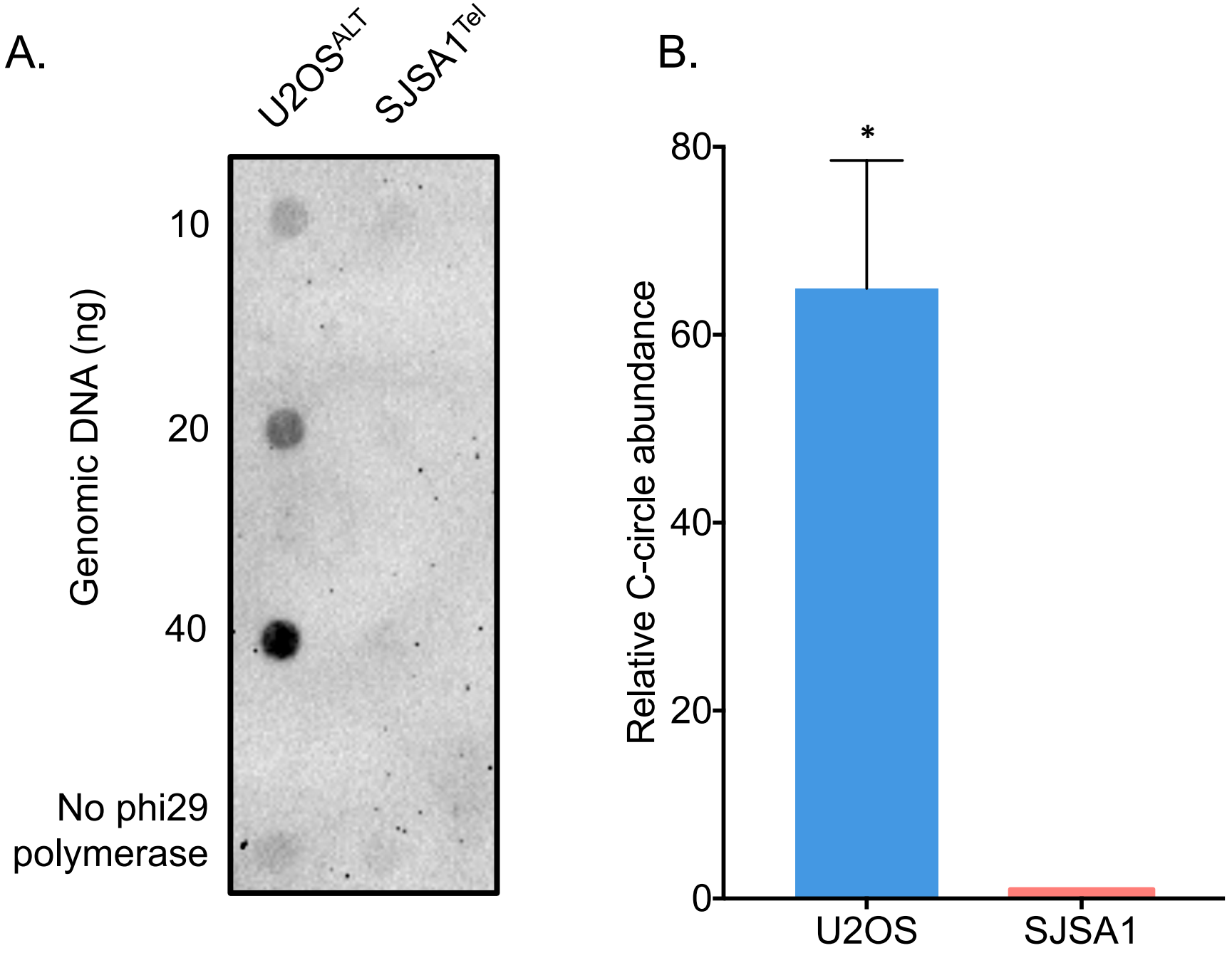


Figure 2



Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Microcentrifuge			Any microcentrifuge will work, eg. Eppendorf 54
Nanodrop	Thermo Scientific	2000	Any DNA quantitation spectrophotometer is suffi
Water bath			A heat block can be used in place of a water bat
Thermocycle			Any thermocycler is sufficient, e.g. BioRad C100
Dot blot apparatus	BioRad	1706545	
UV Crosslinker			Any UV Crosslinker is sufficient, e.g. Boekel Scie
Hybridization bags			Any boilable heat seal pouch will work, e.g. Kapa
Hybridization oven			Any hybridization oven is sufficient, e.g. Thermo
Glass hybridization bottle			Any hybridization bottle that fits the rotisserie in t
Autoclave			
Chemiluminscent imager			Any chemiluminescence imager is sufficient, e.g
Impulse heat sealer			Any impulse heat sealer is sufficient, e.g. FS-305
D-PBS (1X)	Invitrogen	14190-144	Any PBS can be used to wash cell pellet
0.05% Trypsin-EDTA	Thermo Scientific	25300062	Any cell dissociation reagent is sufficient, use re
QIAmp DNA Mini kit	Qiagen	51304	
Alu1	New England Biolabs	R0137L	
Mbo1	New England Biolabs	R0147L	
CutSmart Buffer (10X)	New England Biolabs	B7204S	Buffer is normally supplied with restriction enzym
PureLink Rnase A	Invitrogen	12091-021	
GeneJET PCR Purification Kit	Thermo Scientific	K0701	Qiagen QIAquick PCR purification kit can be use
Bovine Serum Albumin	Sigma	A2153	Any BSA is sufficient
phi29 DNA polymerase	New England Biolabs	MO269L	

phi29 DNA pol	New England		
reaction buffer (10X)	Biolabs	BO269S	Buffer is normally supplied with polymerase
Tween-20	Sigma	P1379	Any Tween 20 is sufficient
dATP solution (100mM)	Invitrogen	55082	
dTTP solution (100mM)	Invitrogen	55085	
dGTP solution (100mM)	Invitrogen	55084	
Sodium Chloride	Sigma	S5886	Any sodium chloride is sufficient
Sodium citrate tribasic dihydrate	Sigma	S4641	
Chromatography paper	Fisherbrand	05-714-4	Any chromatography filter paper is sufficient
Amersham Hybond-XL	GE Healthcare	RPN 203 S	
ULTRAHyb Ultrasensitive Hybridization buffer	Invitrogen	AM8670	Warm in water bath to 65° C before use
Sodium dodecyl sulfate	Fisher Scientific	BP166	Any SDS is sufficient
Telomere probe (5' - CCCTAACCCTAACCC TAACCCTAA - 3')	Invitrogen		25nmoles; standard desalting; resuspend at 250
DIG oligonucleotide 3' - End labeling kit, 2nd generation	Roche	3353575910	Kit contains 5X reaction buffer, CoCl2 solution, D
EDTA	Sigma	6381-92-6	Any EDTA is sufficient
Maleic Acid	Sigma	110-16-7	Any maleic acid is sufficient
Blocking Reagent	Roche	11096176001	
Tris-HCl	Sigma	77-86-1	Any Tris-HCl is sufficient
anti-Digoxigenin-AP Fab fragments	Roche	11093274910	

CDP-Star	Roche	11759051001

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Scientific Shake 'n' Stack

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5 impulse sealer

agent approriate for the cell line

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)IG-ddUTP solution and recombinant terminal transferase

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Author(s): Emily Mason-Osann, Kelli E Cox, Rachel Litman Flynn

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Response to review

Editorial comments:
1.1.4 - how much FBS?
Please see line 131

Prior to peer review, the highlighted portion of your protocol is at our 2.75 page highlighting limit. If additional details are added to the protocol, please adjust the highlighting to identify a total of 2.75 pages of protocol text (which includes sub-headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. The highlighting should include complete statements and not portions of sentences. See JoVE's instructions for authors for more clarification.

•Formatting: Line 129, 134, 137 and others: please consistently use “min” as the standard SI abbreviation throughout, not “mins” or “minutes”. Also, please use “h” for hours and “s” for seconds
We have edited as requested

•Visualization:
-Protocol is discontinuous. Please highlight all steps that are essential for success of the protocol, and unhighlight those that are summary steps without much detail or notes that have no action. For instance, 1.6 and 5.2.2 do not need to be highlighted, and neither does the note in 2.1. However, steps 4.6.6, 4.6.7, 4.9, etc. should be highlighted to maintain continuity.
-Please be careful when highlighting partial steps, e.g. 5.1.7 has some actions that may make more sense to include than to leave out.
We have edited as requested

•Results: Please discuss what the data from the representative results mean in the Results section. What is the interpretation? Also, for Figure 2, range rather than SD should be reported for a sample size of 2.
We have now discussed what the data from the representative results mean in lines 340-342. In addition, we have revised the manuscript to include three independent experiments and reported the extent of the deviation in these experiments as ‘standard deviation’.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript is an informative write-up about the commonly applied method for C-circle detection in mammalian cells. While the method has been published previously (as cited correctly) and is widely used, I am not aware of a step-by-step protocol in the literature, hence this is quite useful. The application is described properly, all the necessary steps are listed correctly and one can expect the correct outcome when following this protocol.

It would be useful to add that the final southern step can well be done with radioactively labeled probes, not just DIG labeled ones.
One small concern is that the authors make a strong statement that C-circles are exclusively observed in ALT cells. That is not entirely correct, as the Pickett lab has observed them also in non-ALT cells with very long telomeres and MEFs. Furthermore, the occurrence of C-circles has been suggested (at the EMBO telomere meeting in Liege, Belgium) in ES cells and IPSC, however, that has not been published yet. Nonetheless, toning down the exclusivity in ALT cells would be advisable.

We truly appreciate the comments and suggestions from Reviewer #1. As suggested, we have toned down the exclusivity of C-circles to ALT cells and welcome the update. In addition, we have added that radioactively labeled probes could also be used to detect the C-Circle amplification products by Southern blot.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #2:

Manuscript Summary:
-Genomic DNA isolation doesn't require the use of any kit as it can easily be achieved by cell lysis/proteinase K digestion followed by ethanol precipitation. Also, clots of DNA can clog the kit columns, thus decreasing the yield.
-As well as, again using a kit after restriction enzyme (RE) digestion can be replaced by ethanol precipitation. Glycogen can be added to help precipitate low amounts of DNA
-RE digestion can be greatly improved by eluting DNA (or re-suspending the DNA pellet) in a master mix containing 1X RE buffer+RE and mixing periodically the samples.
-Post RE digestion, digestion efficiency should be verified by running 1 or 2 µg of DNA on a 1% agarose gel
-Digoxigenin based detection presents several inconveniences: it's expensive and requires alkaline phosphatase chemiluminescent detection that is not quantitative (see kit manufacuter's instruction). Especially if 60 minutes of exposure are required. 32P labeling of the probe would be the most quantitative method but, since radioactivity licenses are not always easily accessible depending on the academic institution, a fluorochrome-conjugated peptide nucleic acid (PNA) probe can also be used.
-The quantification done in Fig2 could have also been done on the 10 and 20ng genomic DNA samples and plotted on a graphic in 2 ways: signal vs amount of genomic DNA and amount per ng genomic DNA vs amount of genomic DNA to ensure that the obtained

values are quantitative and not influenced by the starting amount of genomic DNA (ie linear range of the detection and linear range of Phi29 polymerase)
Reviewer #2 has provided a number of great points regarding the technical approach to the C-circle assay. All of the suggestions are completely accurate and are in fact how we started off doing these assays. Over the years we have begun to modify our protocol to better fit the dynamic of our lab. However, we can appreciate that these points should be included in the manuscript and we have added them in the discussion section.

In Figure 2 we showed the C-circle amplification products from the 10, 20, and 40ng of genomic DNA simply to illustrate that the C-circle products are virtually non-existent in SJSA1 cell lines regardless of the quantity of input genomic DNA. In addition, we wanted to show that there is a significant increase in C-circle abundance in ALT vs. non-ALT cell lines. However, we did not intend to show a thorough and quantitative dynamic range between 10-40ng because as suggested by the reviewer chemiluminescence detection is not ideal for that type of quantitative analysis.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

[Editorial recommendation: Please keep JoVE's protocol requirements in mind as you address the above comments - the protocol must contain sufficient detail in order to enable users to accurately replicate your technique. We recommend NOT removing any details from the protocol text.]