**TITLE:**

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

**AUTHORS:**

Mason-Osann, Emily

Department of Pharmacology & Experimental Therapeutics

Boston University

Boston, MA, USA

[emo@bu.edu](mailto:emo@bu.edu)

Cox, Kelli E

Department of Pharmacology & Experimental Therapeutics

Boston University

Boston, MA, USA

[kellic@bu.edu](mailto:kellic@bu.edu)

Flynn, Rachel Litman

Department of Pharmacology & Experimental Therapeutics

Boston University

Boston, MA, USA

rlflynn@bu.edu

**CORRESPONDING AUTHOR:**

Rachel Litman Flynn

[rlflynn@bu.edu](mailto:rlflynn@bu.edu)

617-638-4346

**KEYWORDS:**

Telomeres, Cancer, Alternative Lengthening of Telomeres, Biomarker, Extra Chromosomal Telomeric Repeats, circular DNA

**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 inhibition5. 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 length3. 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 chromosomes7. 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 factors9. 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 elongation10,11. 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 cells12 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 status11,17. 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. Collect cells from a 10 cm dish with trypsin or by scraping and collect in a microcentrifuge tube.
      1. Wash cells with 3-5 ml of PBS.
      2. If collecting cells by scraping, scrape dish and collect. Otherwise, aspirate PBS from dish.
      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.
      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. 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.
  2. Resuspend the cell pellet in 1 ml of 1X PBS.
  3. 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.
  4. 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 ddH2O.
  5. Measure the genomic DNA concentration using a microvolume spectrophotometer.

1. **Digest genomic DNA** 
   1. Make a restriction digestion master mix with 10 l 10X restriction enzyme buffer, 5 l Alu1 (50 Units), 5 l Mbo1 (25 Units), and 0.2 l 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.

* 1. To digest 15 g of genomic DNA for each sample (more than 15 g of DNA will decrease the efficiency of the digest), mix 15 g of DNA with 20.2 µl of master mix. Bring the total volume of each reaction up to 100 µl with ddH2O and mix thoroughly. Incubate the reaction at 37 °C overnight.
  2. 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.

* 1. Measure the DNA concentration using a microvolume spectrophotometer.

1. **C-circle amplification from digested genomic DNA**
   1. Dilute digested genomic DNA to 10.0 ng/µl in ddH2O to a volume of 250-400 µl.
   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. Prepare a master mix for the amplification reaction such that each reaction contains 10 l of 0.2 mg/ml bovine serum albumin (BSA), 2.5 l of 10X Phi 29 buffer, 0.25 l of 10% Tween-20, and 0.25 l 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.

* 1. 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.
  2. 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 ddH2O.
  3. 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.

1. **Analyze C-circle amplification products using a DNA dot blot**
   1. Prepare 10X SSC buffer.
      1. Prepare a 1 L stock of 20X SSC by adding 175.3 g sodium chloride and 88.2 g sodium citrate to ddH2O. Bring total volume to 1 L with ddH2O.
      2. Dilute 20X SSC by mixing equal volumes 20X SSC and ddH2O.
   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.
   3. Soak the filter papers and nylon membrane in 10X SSC for 10 min at room temperature.
   4. Warm the nucleic acid hybridization buffer to 65 °C in a water bath.
   5. Dilute the amplification reaction products from step 3.6 to 200 µL using 10X SSC.
   6. Set up the dot blot apparatus.
      1. Place two pieces of filter paper on top of the rubber gasket.
      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.
      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. Fill each well with 200 µl of 10X SSC using a multichannel pipette.
      5. Turn on the vacuum to pull the 10X SSC through the membrane.
      6. Tighten the screws while the vacuum is on to ensure a tight seal (do not over tighten).
      7. Turn off the vacuum.
   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.

* 1. 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.
  2. Turn on the vacuum and watch for samples to be pulled through membrane.
  3. 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. Remove the top of the dot blot apparatus and use forceps to peel the membrane off of the filter papers.
  5. Place the membrane on a clean flat surface, with the side containing the sample facing up.
  6. Crosslink the membrane for 35 s in a UV crosslinker (181,540 µJ).
  7. Place the crosslinked membrane inside of a hybridization bag and heat seal the bag on three sides using an impulse heat sealer.
  8. 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.
  9. 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.

1. **Probe dot blot for telomeric sequence**
   1. To detect the C-circle amplification products, label the telomeric (CCCTAA)4 antisense oligonucleotide with digoxigenin following the manufacturer’s instructions.
      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 CoCl2 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 ddH2O.
      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.

* 1. Add 10 µl of the labeled probe (from step 5.1) to 4 ml of fresh hybridization buffer and mix thoroughly with a pipette.
  2. 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.
  3. Gently remove any bubbles from the hybridization bag and reseal the fourth side of the hybridization bag using a heat sealer.
  4. 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. Prepare wash buffers and detection buffers.
     1. Prepare 2X SSC with 0.1% SDS.
     2. Prepare 0.5X SSC with 0.1% SDS.
        1. Warm the 0.5X SSC with 0.1% SDS buffer to 50 °C in a water bath.
     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.
     4. Prepare the 10X maleic acid buffer comprised of 1 M maleic acid and 1.5 M NaCl at pH 7.5.
     5. Prepare the 10X blocking buffer comprised of 10% blocking reagent in 1X maleic acid buffer (prepared from 10X maleic acid buffer diluted in ddH2O).

Note: The 10X blocking buffer should be opaque.

* + - 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.

* + 1. 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 ddH2O unless otherwise stated.

* 1. Remove the membrane from the hybridization bag and place the membrane in a clean container that is slightly larger than the membrane.
  2. 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.
  3. Remove the membrane from the container and place the membrane into a glass hybridization bottle.
  4. 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. Remove the membrane from the glass hybridization bottle and place the membrane in a clean container.
  6. 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.
  7. 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 ddH2O).
  8. 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.
  9. 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).
  10. Incubate the membrane with the anti-DIG-AP in blocking buffer for 30 min at room temperature while gently shaking.
  11. 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.
  12. Place the membrane on a clean flat surface and equilibrate the membrane in 9 ml of 1X detection buffer.
  13. Dilute the alkaline phosphatase chemiluminescent substrate to 0.25 mM in 1 ml of detection buffer.
  14. 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.
  15. 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 pathway3. 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 tumors15,18,19. 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 amplification17. 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.

**ACKNOWLEDGMENTS:**

We thank members of the R.L.F. lab for review of the manuscript. R.L.F. is supported by the NIH Pathway to Independence Award (CA166729), the Karin Grunebaum Cancer Research Foundation, Peter Paul Professorship, and the Edward Mallinckrodt Jr. Foundation. E.M.O was also supported by the NIGMS T32 funded Program in Biomolecular Pharmacology (3T32GM008541-18S1).

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES**

1. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, (5), 646–74, doi:10.1016/j.cell.2011.02.013 (2011).

2. Shay, J. W. & Bacchetti, S. A survey of telomerase activity in human cancer. *Eur J Cancer* **33** (5), 787–91, doi:10.1016/S0959-8049(97)00062-2 (1997).

3. Bryan, T. M., Englezou, A., Gupta, J., Bacchetti, S. & Reddel, R. R. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* **14** (17), 4240–8at (1995).

4. Ouellette, M. M., Wright, W. E. & Shay, J. W. Targeting telomerase-expressing cancer cells. *J Cell Mol Med* **15** (7), 1433–42, doi:10.1111/j.1582-4934.2011.01279.x (2011).

5. Hu, J., Hwang, S. S., *et al.* Antitelomerase therapy provokes ALT and mitochondrial adaptive mechanisms in cancer. *Cell* **148** (4), 651–63, doi:10.1016/j.cell.2011.12.028 (2012).

6. Flynn, R. L., Cox, K. E., *et al.* Alternative lengthening of telomeres renders cancer cells hypersensitive to ATR inhibitors. *Science*  **347** (6219) 273–7 (2015).

7. Dunham, M. A., Neumann, A. A., Fasching, C. L. & Reddel, R. R. Telomere maintenance by recombination in human cells. *Nature Genet* **26** (4), 447–50, doi:10.1038/82586 (2000).

8. Varley, H., Pickett, H. a, Foxon, J. L., Reddel, R. R. & Royle, N. J. Molecular characterization of inter-telomere and intra-telomere mutations in human ALT cells. *Nature Genet* **30** (3), 301–5, doi:10.1038/ng834 (2002).

9. Yeager, T. R., Neumann, A. A., *et al.* Telomerase-negative Immortalized Human Cells Contain a Novel Type of Promyelocytic Leukemia ( PML ) Body. *Cancer Res* **59** (17), *4175*–4179 (1999).

10. Tokutake, Y., Matsumoto, T., *et al.* Extra-Chromosomal Telomere Repeat DNA in telomerase-negative immortalized cell lines. *Biochem Biophys Res Commun* **247** (3), 765–772 (1998).

11. Nabetani, A. & Ishikawa, F. Unusual telomeric DNAs in human telomerase-negative immortalized cells. *Mol Cell Biol* **29** (3), 703–13, doi:10.1128/MCB.00603-08 (2009).

12. Pickett, H. a, Cesare, A. J., Johnston, R. L., Neumann, A. a & Reddel, R. R. Control of telomere length by a trimming mechanism that involves generation of t-circles. *EMBO J* **28** (7), 799–809, doi:10.1038/emboj.2009.42 (2009).

13. Cerone, M. A., Autexier, C., Londoño-Vallejo, J. A. & Bacchetti, S. A human cell line that maintains telomeres in the absence of telomerase and of key markers of ALT. *Oncogene* **24** (53), 7893–7901, doi:10.1038/sj.onc.1208934 (2005).

14. Yeager, T. R., Neumann, A. A., Englezou, A., Huschtscha, L. I., Noble, J. R. & Reddel, R. R. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Res* **59** (17), 4175–9 (1999).

15. Henson, J. D., *et al.* A Robust Assay for Alternative Lengthening of Telomeres in Tumors Shows the Significance of Alternative Lengthening of Telomeres in Sarcomas and Astrocytomas. *Clin Cancer Res* **1**(11) 217–225 (2005).

16. Cesare, A. J. & Reddel, R. R. Alternative lengthening of telomeres: models, mechanisms and implications. *Nature Rev Genet* **11** (5), 319–30, doi:10.1038/nrg2763 (2010).

17. Henson, J. D., Cao, Y., *et al.* DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. *Nature Biotechnol* **27** (12), 1181–5, doi:10.1038/nbt.1587 (2009).

18. Heaphy, C. M., de Wilde, R. F., *et al.* Altered telomeres in tumors with ATRX and DAXX mutations. *Science* **333** (6041), 425, doi:10.1126/science.1207313 (2011).

19. Heaphy, C. M., Subhawong, A. P., *et al.* Prevalence of the alternative lengthening of telomeres telomere maintenance mechanism in human cancer subtypes. *Am J Pathol* **179** (4), 1608–15, doi:10.1016/j.ajpath.2011.06.018 (2011).

20. Lo, Y. M. Circulating nucleic acids in plasma and serum: an overview. *Ann N Y Acad Sci* **945**, 1–7 (2001).