**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.]