Benchmarks

Nonradioactive method to detect native single-stranded G-tails on yeast telomeres using a modified Southern blot protocol

Lina M. Ortega, Christoph J. Hengartner, and Leticia R. Vega
*Department of Biology, College of Arts and Sciences, Barry University,
 Miami Shores, FL, USA*

BioTechniques 50:407–410 (June 2011) doi 10.2144/000113687

Keywords: Telomere; nonradioactive; G-tail; yeast; *Saccharomyces cerevisiae*

Supplementary material for this article is available at www.BioTechniques.com/article/113687.

Because of their low abundance and short length, telomeric single-stranded extensions have not traditionally been assessed by Southern blot analysis. Instead, most methods have relied on hybridizing radioactively labeled oligonucleotide probes to electrophoresed DNA within agarose gels. Here we describe a rapid and nonradioactive Southern blot–derived method to transfer and detect telomeric single-stranded G-rich overhangs (G-tails) under nondenaturing (native) conditions, using *Saccharomyces cerevisiae* DNA. Restriction enzyme–digested chromosomal DNA is separated by agarose gel electrophoresis, transferred onto a charged membrane by electroblotting under nondenaturing conditions, and probed with a digoxigenin (DIG)–labeled oligonucleotide. Compared with the prolonged film exposure required to detect radioactive probes, detection of short single-strand G-tails with this method takes mere minutes. Furthermore, following detection of the single-stranded G-tails, the DNA on the membrane can be denatured and reprobed using conventional hybridization and detection methods.

Telomeres facilitate the replication and maintenance of linear eukaryotic chromosomes. Telomeres consist of stretches of repeated CG-rich DNA ending with 3' single-stranded G-rich overhang (G-tail). In the baker's yeast, *Saccharomyces cerevisiae*, chromosomes end in ~300 bp of C₁₋₃A/TG₁₋₃ DNA, followed by ~10–15 bases of single-stranded G-tail. The length of these telomeric G-tails changes little throughout most of the yeast cell cycle, with the exception of a transient increase in late S/G2 phase (1,2). In both yeast and higher eukaryotes, proteins bind and organize telomeric DNA into a nonnucleosomal chromatin structure called the telosome (3). In *S. cerevisiae*, the essential

telosome protein Cdc13p binds to single-stranded G-tails (4) and is required to protect telomeric DNA from degradation (5). Another yeast telosome component, the heterodimeric Ku complex, plays many important roles, including protecting telomeres from degradation and inappropriate recombination (6–8). Yeast cells limited for Cdc13p or for the Ku complex have uncapped telomeres and exhibit longer single-stranded G-tails (5,9).

Determination of the telomeric 3' single-strand overhangs is a measure of telomere dysfunction. Several methods to detect and assess the size of single-stranded G-tails on native telomeres have been described, including: (i) hybridization

with a radioactive oligonucleotide probe in-gel following electrophoresis (2,10); (ii) hybridization of a radioactive oligonucleotide to native DNA prior to agarose gel electrophoresis (11); (iii) telomere-oligonucleotide ligation assay (T-OLA) (12); (iv) quantitative amplification of single-stranded DNA (QAOS) (13); (v) G-tail telomere hybridization protection assay (14); (vi) overhang protection assay (15); and recently, (vii) quantitative telomeric overhang determination using a double-strand–specific nuclease (16). The most commonly used method to examine short 3' overhangs in yeast involves the use of radioactive oligonucleotide probe labeled to a high specific activity to hybridize with the telomeric single-stranded G-tails in-gel (2). Previous attempts to tailor the Southern blotting method for telomeric overhang detection yielded poor results. For example, treating gels with HCl prior to transfer improves transfer efficiency by depurinating DNA fragments into smaller pieces; however, such a treatment may potentially compromise the integrity of the short G-tails. Alkaline transfer methods denature DNA and enhance binding to the support membrane, but DNA denaturation will prevent the specific detection of the single-stranded telomeric overhangs (17). UV crosslinking of fragment DNA to nylon membranes permanently fixes the DNA onto the surface, but it may compromise single-strand G-tails detection. Indeed, UV crosslinking was shown to reduce the ability of a probe to hybridize to its target DNA (17).

Our goal was to adapt the Southern blot protocol for successful telomeric overhang detection on an immobilized support using a nonradioactive probe. We designed a custom digoxigenin (DIG)–labeled oligonucleotide to detect short single-stranded G-tails at native yeast telomeres, and we modified existing methods to efficiently transfer the native DNA onto a charged membrane. Not only could single-stranded G-tails be detected under nondenaturing conditions, but DNA on the membrane could also be denatured and successfully reprobed under traditional Southern blot hybridization.

To validate our procedure, we examined the telomere end structure of a wild-type yeast strain (YPH499) carrying normal G-tails and two mutant yeast strains (*yku70Δ* and *cdc13-1*) with aberrant G-tails (Figure 1). The *yku70Δ* cells exhibit constitutively long, single-stranded G-tails (9), while the *cdc13-1* cells show normal G-tails at the permissive temperature (23°C) but exhibit a lengthening of the single-stranded regions at

Figure 1. Southern blot analysis detection of single-stranded G-tails on yeast telomeres. Genomic DNA from the indicated strains was prepared as described (2,11,18). Prior to *XhoI* restriction enzyme digestion, gel electrophoresis, and Southern blot analysis, genomic DNA was either digested with *E. coli* exonuclease I (+) to remove single-stranded telomeric G-tail overhangs or was left untreated (-). (A) Native DNA probed with DIG-labeled oligonucleotide. Signal strength reflects relative length of single-stranded G-tail overhangs. (B) DNA on the membrane was denatured with 0.4 N NaOH and reprobbed with a telomeric probe fragment. Marker, DNA Molecular Weight Marker VII, DIG-labeled (Roche Applied Biosciences). (C) Native telomeric restriction fragment (TRF) signal/denatured TRF signal ratio normalized to wild-type (WT) quantified with Quantity One software (Bio-Rad Laboratories). Error bars indicate the SEM of three trials for exonuclease I (+) or four trials for exonuclease I (-).

Figure 2. Signal response to varying DNA amounts. To determine the limits of detection of our method, varying amounts of genomic DNA from wild-type (WT; lanes 2–5) or *yku70Δ* (lanes 6–9) strains prepared as before, with the exception that none of the samples were digested with *E. coli* exonuclease I: 60 μg (lanes 4–7), 40 μg (lanes 3 and 8), and 20 μg (lanes 2 and 9); 20 ng DIG-labeled DNA Molecular Weight Marker VII (Roche-Applied Sciences) (lane 1). (A) G-tail signal from *XhoI*-digested native DNA. (B) Telomere signal from the reprobbed denatured blot. (C) Relative intensities of the telomeric restriction fragment for native (open symbols) and denatured (filled symbols) blots using the Quantity One software. The data for the two 60-μg lanes were averaged.

the elevated temperature (37°C) (5). All three strains were grown in 1% yeast extract, 2% peptone, and 2% dextrose (YPD) with 2% glucose at the indicated temperatures until the cell density reached $1-2 \times 10^7$ cells/mL ($OD_{600} = 0.4$ U), and genomic DNA was isolated as previously described (2,11,18). Following DNA extraction, genomic DNA was treated with RNase A (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C, ethanol-precipitated, and dissolved in 50 μL 1× Tris-EDTA (TE). Sixty micrograms genomic DNA were then either treated (+) with 50 U *Escherichia coli* exonuclease

I (Epicenter Biotechnologies, Madison, WI, USA) or mock-treated (-) for 2 h at 37°C. Exonuclease-treated (+) genomic DNA will have lost its single-stranded telomeric G-tails, while the mock-treated genomic DNA (-) will still retain them. After exonuclease I digestion, the DNA was ethanol-precipitated, washed in 70% ethanol, and digested with 40 U *XhoI* (New England Biolabs, Ipswich, MA, USA) in 200 μL 1× NEB Buffer 4 (New England Biolabs). The *XhoI*-digested DNA was then precipitated and resuspended in 15 μL 1× TE overnight at 4°C. The DNA was electrophoresed on a 12 cm × 14 cm Owl

EasyCast Mini Gel System (Thermo Scientific, Portsmouth, NH, USA) in a 0.8% agarose gel containing 0.5× Tris-Borate-EDTA (TBE) without ethidium bromide (EtBr) for 18 h at 22 V. The native DNA fragments were then transferred at 117 mA (0.81 mA/cm²) for 120 min onto a Hybond N+ membrane (GE Healthcare Life Sciences, Piscataway, NJ USA) using a Trans-Blot Semi-Dry electrophoretic transfer cell and a Trans-Blot SD DNA gel support frame (Bio-Rad Laboratories, Hercules, CA, USA) at 4°C in 0.5× TBE. Transfer conditions were empirically determined as suggested by the manufac-

turer in the Trans-Blot SD manual. While low-molecular weight DNA transfer out of agarose gels efficiently, transfer of the higher-molecular weight DNA requires additional fine tuning. We found that the high voltages needed to transfer native high molecular DNA to charged membranes at the low ionic strength of the transfer buffer could result in denaturation of genomic DNA due to increased heat, thus a slower transfer was used. After the transfer was complete, the agarose gel was stained with 0.5 $\mu\text{g/mL}$ EtBr and photographed to monitor the efficiency of DNA transfer. We did not directly evaluate the efficiency of DNA retention on the Hybond N+ membrane compared with tradition blotting by capillary action. The transferred DNA was crosslinked to the membrane in a UV crosslinker set at autocrosslink (VWR, Radnor, PA USA). The membrane was prehybridized for a minimum of 30 min at 35°C with DIG granules as recommended by the manufacturer of the DIG High Prime DNA Labeling and Detection Starter kit II (Roche Applied Sciences, Mannheim, Germany). The membrane was then hybridized overnight at 35°C with 10 nM custom oligonucleotide containing the following three nucleotides modified with DIG via a C6 amino linker: (i) the 5' terminal nucleotide, (ii) the 3' terminal nucleotide, and (iii) an internal dA at position 11 (CCCACCCACAC[Am dA-C6]CaCCCACACCC; Gene Link, Hawthorne, NY, USA). The rationale for choosing these positions is that we wanted to increase the sensitivity of the probe while minimizing steric hindrance of the labeled bases by spacing them apart ≥ 10 bases. Following hybridization, the membrane was washed twice for 5 min in $2\times \text{SSC}/0.1\%$ SDS at room temperature and twice for 5 min in $0.5\times \text{SSC}/0.1\%$ SDS at 37°C. Probe-bound DNA fragments carrying telomeric single-stranded G-tails were detected using the DIG High Prime DNA Labeling and Detection Starter kit II and developing an Amersham Hyperfilm ECL (GE Healthcare Life Sciences) that was exposed (Figure 1A) for 15 min to the probed membrane. As expected, the G-tail signal was lost or greatly reduced when genomic DNA was treated with *E. coli* exonuclease I (Figure 1A, lanes 3, 5, 7, and 9).

Following detection of the G-tails, the blot was placed on extra thick blotting paper (Bio-Rad Laboratories) saturated with 0.4 N NaOH for 5 min at room temperature to denature the DNA (17). The membrane was rinsed briefly in $2\times \text{SSC}$ at room temperature, prehybridized

with DIG High Prime DNA hybridization granules according to the manufacture at 42°C for 5 h, and hybridized at 42°C overnight with a double-stranded DIG labeled C_{1-3}A telomere probe to detect bulk telomeres (Figure 1B). To prepare the bulk telomere probe, an *EcoRI/KpnI* fragment from pCT300 (2) was gel-purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Twenty-five nanograms of template DNA were labeled by PCR using 4 μL DIG High Prime Vial 1 and 1 μL 5 U/ μL *Taq* DNA polymerase (New England Biolabs) in a final volume of 30 μL $1\times \text{Taq}$ Buffer (New England Biolabs). The PCR conditions consisted of an initial denaturation step of 4 min at 95°C; followed by 30 cycles of alternating 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step of 7 min at 72°C. The PCR product was denatured at 95°C for 5 min and added to the hybridization solution from the DIG High Prime kit. The bulk telomere probe could be stored in this form at -20°C for up to 1 year and could be used for multiple hybridizations. Prior to reusing the telomere probe, the probe and hybridization mixture were incubated at 65°C for 5 min to denature the double-stranded telomere probe. Probe detection followed the protocol of the manufacturer of the DIG High Prime DNA Labeling and Detection Starter Kit II, and the results could be visualized after developing an Amersham Hyperfilm ECL that was briefly exposed to the probed membrane.

To monitor the sensitivity of our assay, varying amounts of DNA from wild-type or *yku70 Δ* strains were processed and probed as described above. As little as 20 μg wild-type DNA are able to produce a single-strand G-tail signal after a 15-min exposure (Figure 2A, lane 2); however, we recommend 40–60 μg DNA. The major advantage of detecting single-stranded G-tails on native DNA using the method we describe here is that radioisotopes are no longer needed; this simplifies the overall procedure and reduces the costs. We anticipate the blotting technique described in this study to transfer native DNA is likely to be compatible with the use of a radiolabeled oligonucleotide probe. Furthermore, this method produces faster results than in-gel methods; short single-strand G-tails are detected after 15-min film exposure rather than days. Moreover, since the DNA is immobilized onto a solid substrate, the membrane may be stripped and reprobed at a later time. Finally, the DIG oligonucleotide probe described in this study is likely to be useful in other methods to examine single-stranded telomeric overhangs, including

Power and Performance at your fingertips

ExpressGene™ Gradient Cycler

- Live sensor feedback for superior temperature control
- 96 well gradient thermal cycler
- Large graphical touch screen user interface
- Rapid intuitive “drag and drop” program creation

Only \$3,995

DENVILLE
SCIENTIFIC INC.

800.453.0385 | www.densci.com

NEW!

Precise, Light & Dependable

BRAND HandyStep® S Repeating Pipette

The NEW HandyStep® S provides unprecedented versatility in a mechanical repeating pipette, with accuracy, precision and dependability.

- Comfortable, light weight, and well balanced
- Easy one handed volume selection
- Flexible; accepts third party tips – no more being locked into a sole source
- Tip ejection system allows tip removal without user handling.

Economical
BRAND PD-Tip™
syringe tips fit all
repeating pipettes
using non-proprietary
designs.

Looking for an electronic
repeating pipette?

Try the rapid, relaxed
and reliable HandyStep®
electronic.

Lab Rats Trust BrandTech!

BRANDTECH
SCIENTIFIC, INC.

Toll Free (888) 522-2726
www.brandtech.com

the quantitative telomeric overhang determination using a double-strand specific nuclease (16).

Acknowledgments

We thank Alison Bertuch for providing protocols and helpful discussions, Virginia Zakian for providing us with pCT300, Ali Javed from Gene Link for help with the oligonucleotide design, and Frank Solomon for helpful comments and suggestions. This work was supported by funds from Barry University's School of Natural and Health Sciences Research Scholarship Funds and the National Institutes of Health-National Institute of General Medical Sciences/National Cancer Institute (NIH-NIGMS/NCI) Minority Biomedical Research Support (MBRS) Support for Continuous Research Excellence (SCORE) grant no. 5SC2CA 138567, and grant no. 3 SC2 CA138567-02S1 (to L.R.V.). L.M.O. was supported by NIH-NIGMS Minority Access to Research Careers (MARC) grant no. 5T34 GM08021 and NIH-NIGMS MBRS Research Initiative for Scientific Enhancement (RISE) grant no. 5R25 GM059244, Barry University. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCI or the NIH. This paper is subject to the NIH Public Access Policy.

Competing interests

The authors declare no competing interests.

References

1. Larrivee, M., C. LeBel, and R.J. Wellinger. 2004. The generation of proper constitutive G-tails on yeast telomeres is dependent on the MRX complex. *Genes Dev.* 18:1391-1396.
2. Wellinger, R.J., A.J. Wolf, and V.A. Zakian. 1993. *Saccharomyces* telomeres acquire single-strand TG1-3 tails late in S phase. *Cell* 72:51-60.
3. Wright, J.H., D.E. Gottschling, and V.A. Zakian. 1992. *Saccharomyces* telomeres assume a non-nucleosomal chromatin structure. *Genes Dev.* 6:197-210.
4. Bourns, B.D., M.K. Alexander, A.M. Smith, and V.A. Zakian. 1998. Sir proteins, Rif proteins, and Cdc13p bind *Saccharomyces* telomeres in vivo. *Mol. Cell. Biol.* 18:5600-5608.
5. Garvik, B., M. Carson, and L. Hartwell. 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. *Mol. Cell. Biol.* 15:6128-6138.
6. DuBois, M.L., Z.W. Haimberger, M.W. McIntosh, and D.E. Gottschling. 2002. A quantitative assay for telomere protection in *Saccharomyces cerevisiae*. *Genetics* 161:995-1013.
7. Maringele, L. and D. Lydall. 2002. EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Delta mutants. *Genes Dev.* 16:1919-1933.
8. Polotnianka, R.M., J. Li, and A.J. Lustig. 1998. The yeast Ku heterodimer is essential for protection of the telomere against nucleolytic and recombinational activities. *Curr. Biol.* 8:831-834.
9. Gravel, S., M. Larrivee, P. Labrecque, and R.J. Wellinger. 1998. Yeast Ku as a regulator of chromosomal DNA end structure. *Science* 280:741-744.
10. Dionne, I. and R.J. Wellinger. 1996. Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. *Proc. Natl. Acad. Sci. USA* 93:13902-13907.
11. Bertuch, A.A. and V. Lundblad. 2003. The Ku heterodimer performs separable activities at double-strand breaks and chromosome termini. *Mol. Cell. Biol.* 23:8202-8215.
12. Cimino-Reale, G., E. Pascale, E. Battiloro, G. Starace, R. Verna, and E. D'Ambrosio. 2001. The length of telomeric G-rich strand 3'-overhang measured by oligonucleotide ligation assay. *Nucleic Acids Res.* 29:E35.
13. Booth, C., E. Griffith, G. Brady, and D. Lydall. 2001. Quantitative amplification of single-stranded DNA (QAOS) demonstrates that cdc13-1 mutants generate ssDNA in a telomere to centromere direction. *Nucleic Acids Res.* 29:4414-4422.
14. Tahara, H., M. Kusunoki, Y. Yamanaka, S. Matsumura, and T. Ide. 2005. G-tail telomere HPA: simple measurement of human single-stranded telomeric overhangs. *Nat. Methods* 2:829-831.
15. Chai, W., J.W. Shay, and W.E. Wright. 2005. Human telomeres maintain their overhang length at senescence. *Mol. Cell. Biol.* 25:2158-2168.
16. Zhao, Y., H. Hoshiyama, J.W. Shay, and W.E. Wright. 2008. Quantitative telomeric overhang determination using a double-strand specific nuclease. *Nucleic Acids Res.* 36:e14.
17. Reed, K.C. and D.A. Mann. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* 13:7207-7221.
18. Hoffman, C.S. and F. Winston. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* 57:267-272.

Received 3 June 2010; accepted 4 April 2011.

Address correspondence to Leticia R. Vega, Barry University, 11300 NE 2nd Ave, Miami Shores, FL 33161, USA. e-mail: lvega@mail.barry.edu

To purchase reprints of this article, contact:
biotechniques@fosterprinting.com